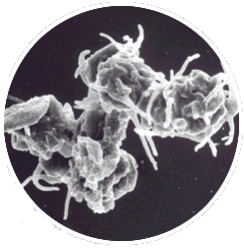
Citometría de flujo aplicada al diagnóstico de trombocitopatías



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Academia Nacional de Medicina de Buenos Aires
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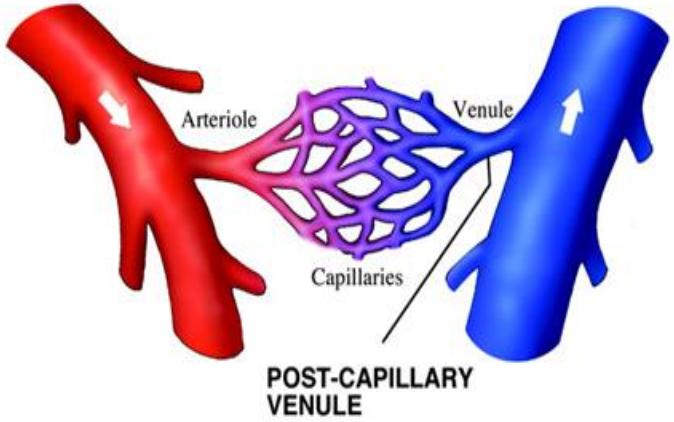
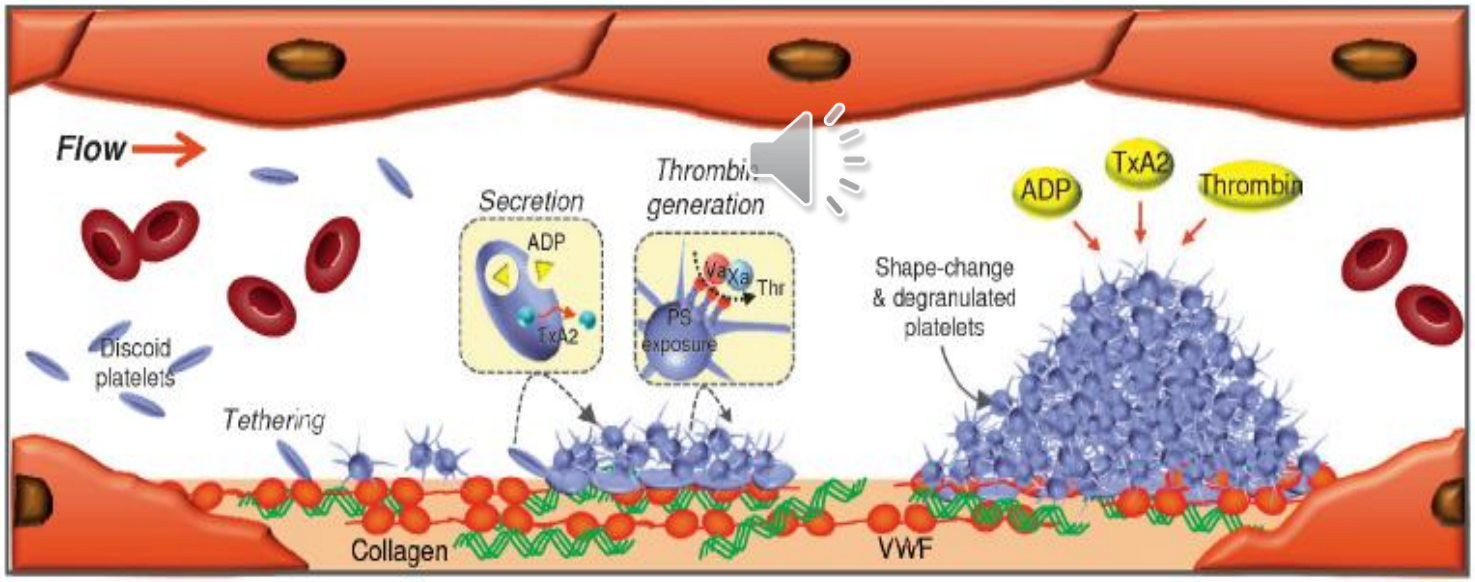
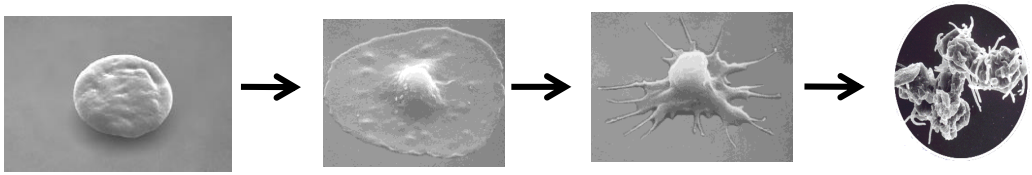
GRUPO RIOPLATENSE DE CITOMETRIA DE FLUJO

Respuesta plaquetaria a la injuria

Iniciación: captura, adhesión, activación
Extensión: cohesión y secreción
Perpetuación: estabilización del trombo

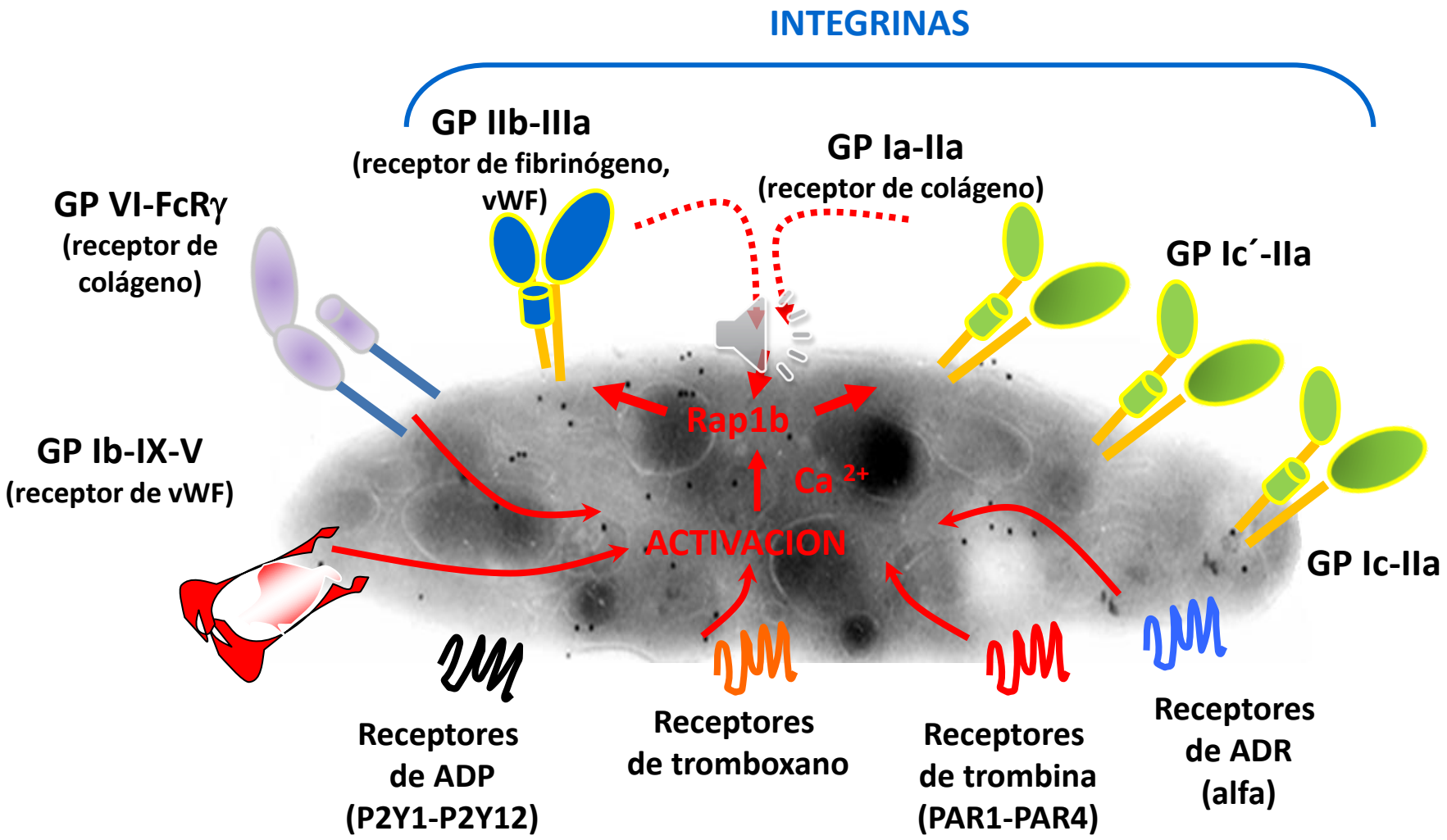






McFadyen JD, 2013

Receptores que median la activación: glicoproteínas de membrana y receptores acoplados a proteína G



La función plaquetaria puede estar disminuida por alteraciones a nivel:

- de los receptores para proteínas adhesivas

- de los receptores para agonistas solubles

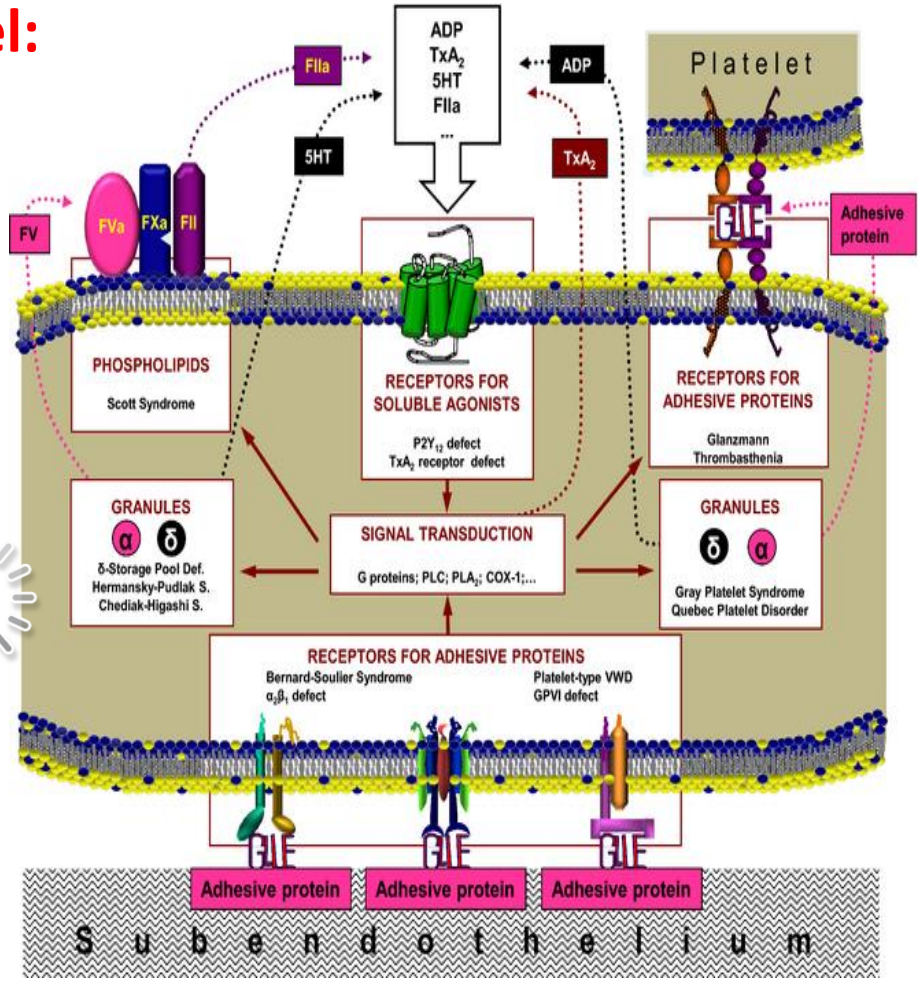
- de los gránulos de secreción

- de los mecanismos de transducción de señal

- de los fosfolípidos pro-coagulantes

- de las proteínas del citoesqueleto

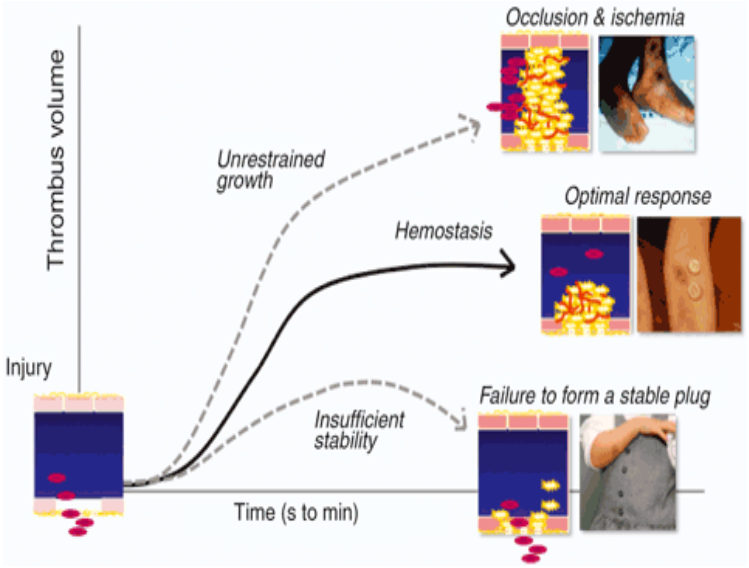
- misceláneas, desórdenes no caracterizados



Dovlatova N, 2015

Los métodos destinados a evaluar la función plaquetaria fueron desarrollados inicialmente para tratar de identificar alteraciones que se relacionaran a la clínica de sangrado de los pacientes.

Actualmente disponemos de una variedad de métodos que se adaptan a nuevos contextos tales como:



Brass LF, 2011

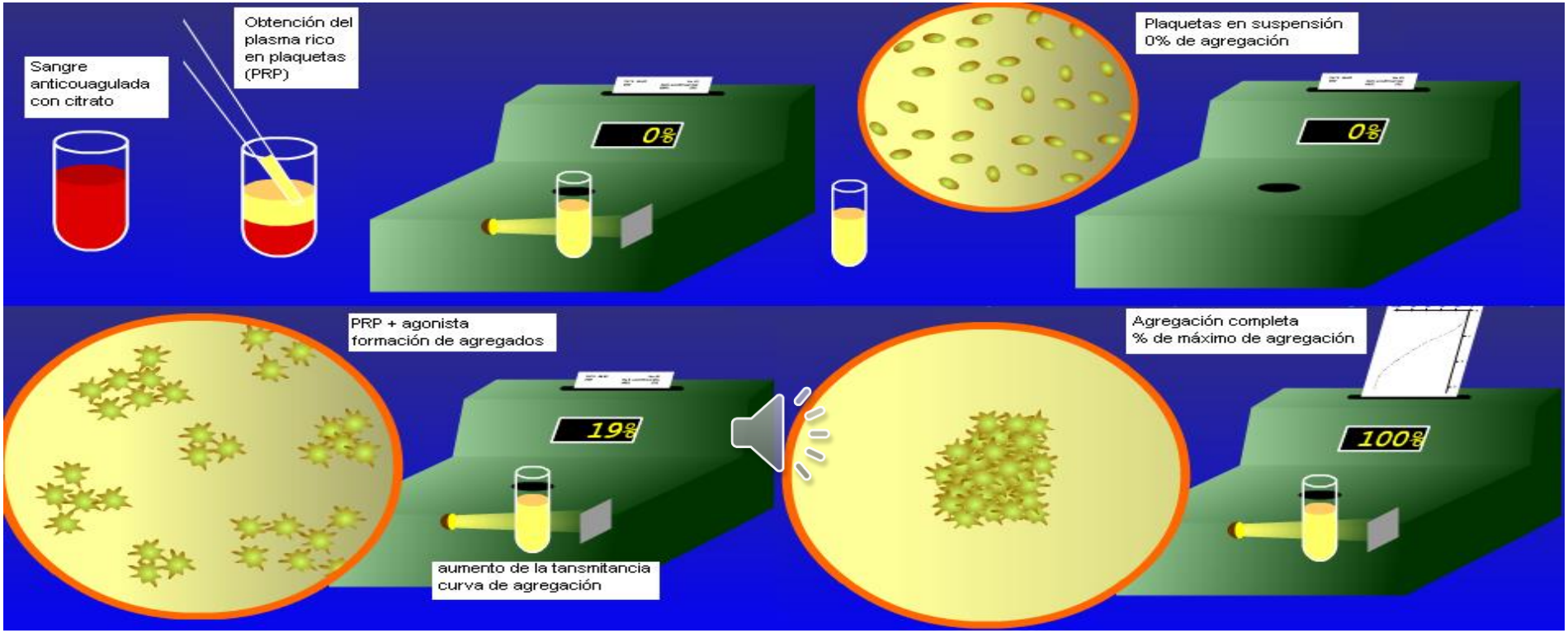
- la investigación básica sobre fisiología plaquetaria,

- las asociaciones fenotipo-genotipo en trastornos hemorrágicos,

- el desarrollo farmacológico como criterios indirectos de eficacia de la nueva terapia antiplaquetaria,

- la monitorización de la terapia antiagregante en la práctica clínica, para predecir el riesgo trombótico y hemorrágico.

Agregación plaquetaria por método óptico: método de referencia para el estudio de la función plaquetaria


Sangre anticouagulada con citrato
Obtención del plasma rico en plaquetas (PRP)
0%
Plaquetas en suspensión 0% de agregación
0%
PRP + agonista formación de agregados
19%
aumento de la tansmitancia curva de agregación
Agregación completa % de máximo de agregación
100%

Agonistas de uso común:

ADP (2.5-5.0 µM)

ADR (10.0 µM)

Colágeno (1.0 µg/ml)

Acido Araquidónico (0.5 mM)

Ristocetina (0.5-0.7 y 1.2 mg/ml)

Agonistas especiales:

TRAP (10.0 µM)

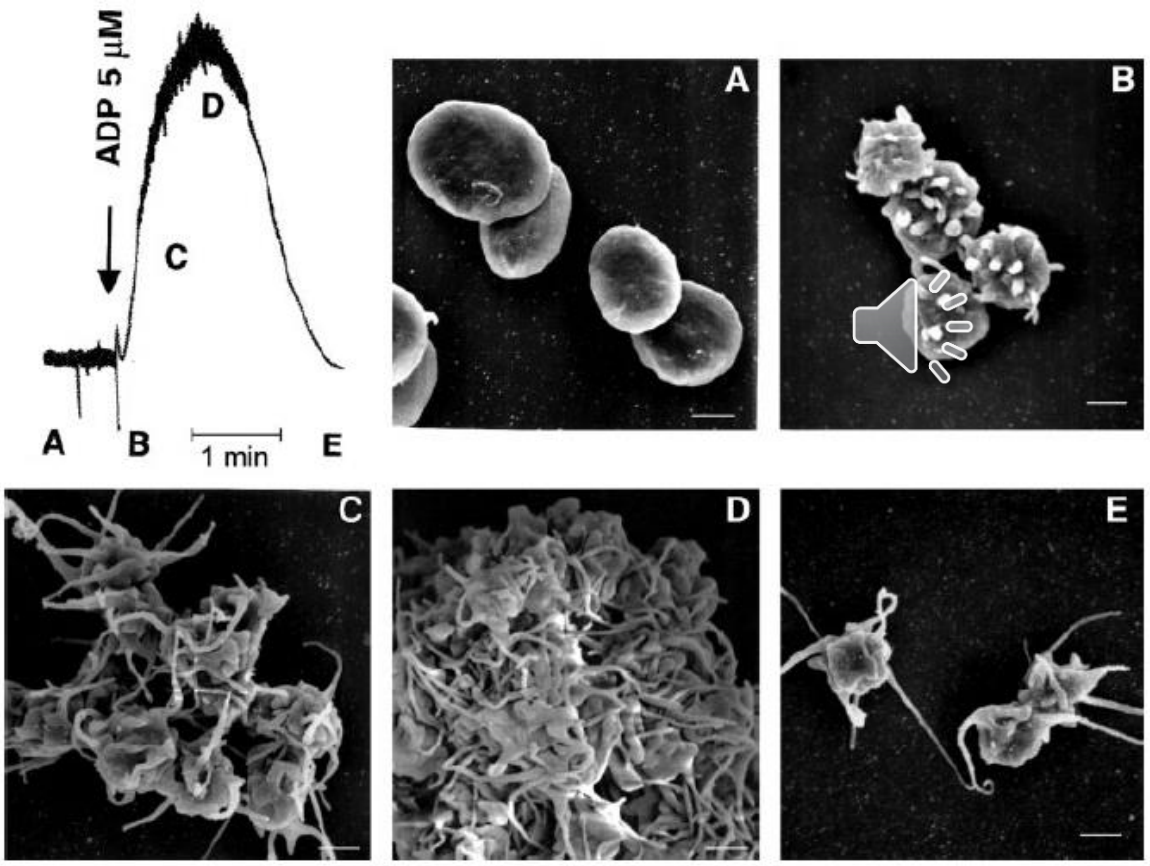
Análogo del tromboxano U46619 (1.0 µM)

Ionóforo de calcio (1.0 µM)

PMA (1.0 µM)

Agregación plaquetaria por método óptico:

La información cinética de las interacciones plaqueta-plaqueta corresponde a fases específicas de la activación desde el cambio de forma, la degranulación y la agregación plaquetaria firme o inestable, confirmada por microscopía electrónica.

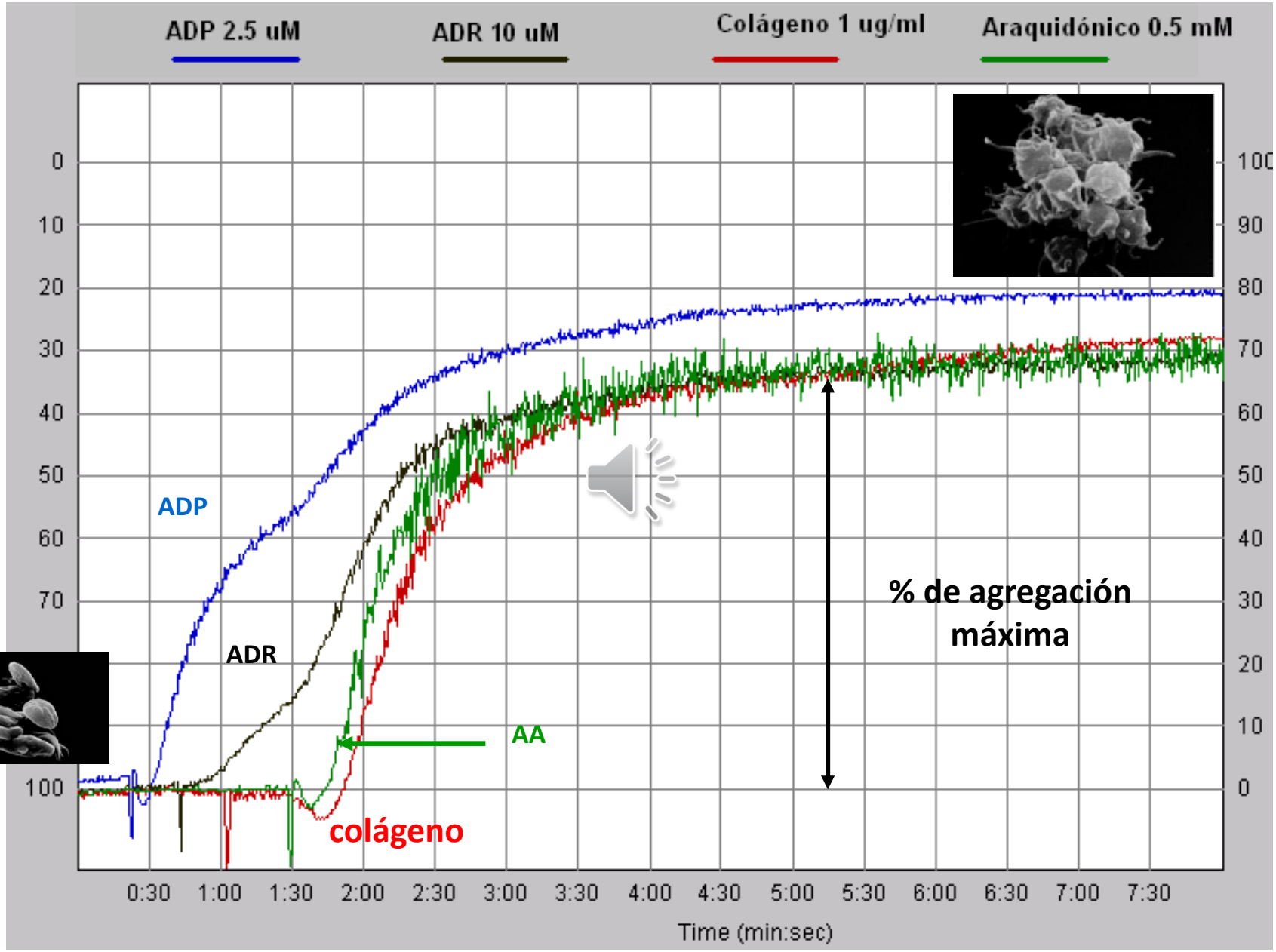


Cambios morfológicos de las plaquetas en la agregación con ADP:

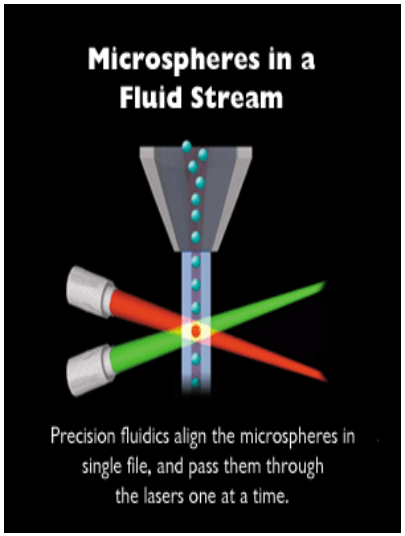
Las plaquetas fueron fijadas a diferentes tiempos para ser visualizadas por microscopía electrónica.

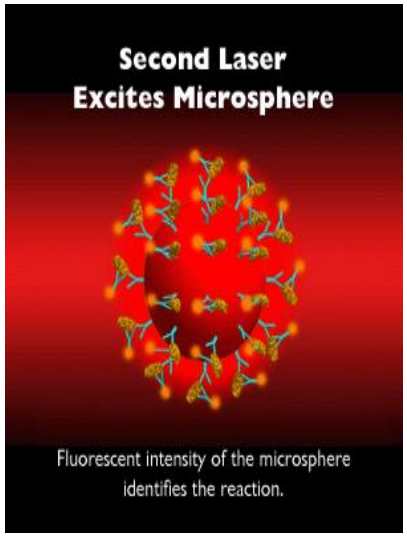
A) Plaquetas discoideas en estado de reposo.
B) Cambio de forma y formación de seudópodos tempranos (7 segundos).
C) Primeras interacciones plaqueta-plaqueta (20 segundos).
D) Agregados plaquetarios grandes (45 segundos).
E) Plaquetas aisladas luego de desagregación (3 minutos).

Lordkipanidzé M, 2016


ADP 2.5 uM
ADR 10 uM
Colágeno 1 ug/ml
Araquidónico 0.5 mM
0
10
20
30
40
50
60
70
100
100
90
80
70
60
50
40
30
20
10
0
ADP
ADR
AA
colágeno
% de agregación máxima
0:30
1:00
1:30
2:00
2:30
3:00
3:30
4:00
4:30
5:00
5:30
6:00
6:30
7:00
7:30
Time (min:sec)

CITOMETRIA DE FLUJO


Microspheres in a
Fluid Stream
Precision fluidics align the microspheres in
single file, and pass them through
the lasers one at a time.



Plaquetas sin estimular

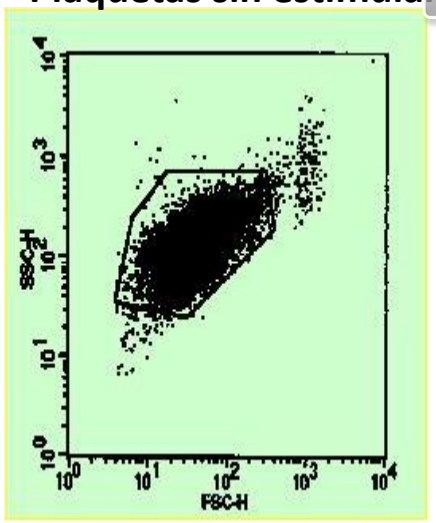
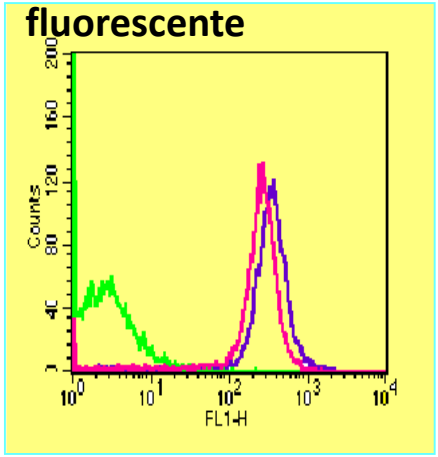


Grafico de puntos (dot-plot) dispersión frontal vs dispersión lateral

Marcador fluorescente



Histograma, intensidad de fluorescencia media

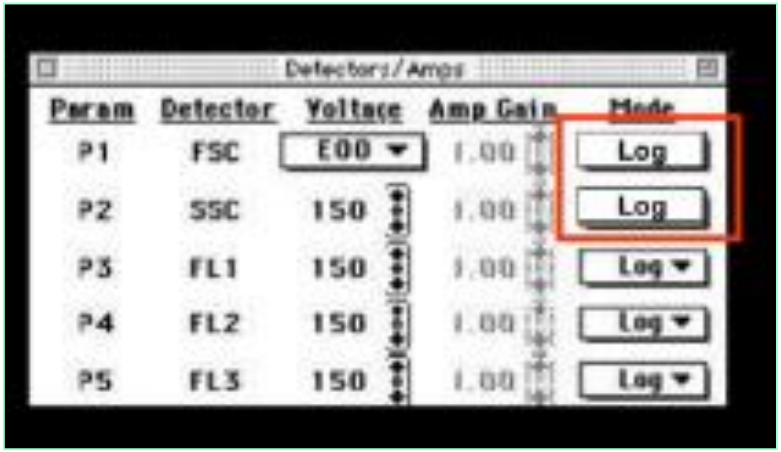
AJUSTES BÁSICOS DEL SOFTWARE

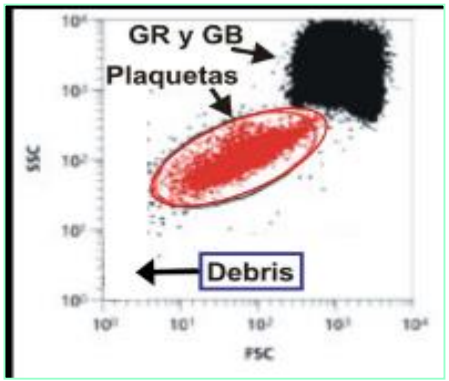
ADQUISICIÓN

10000 eventos

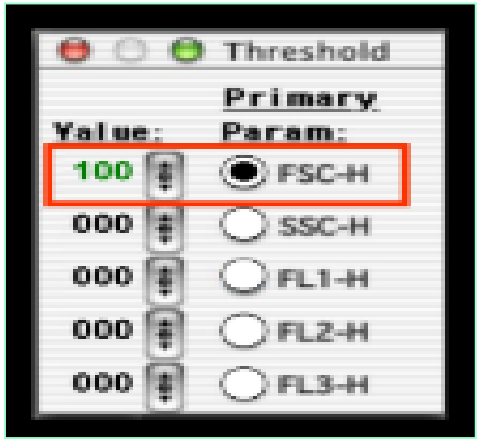
CELL-QUEST

ESCALA DE FCS/SSC



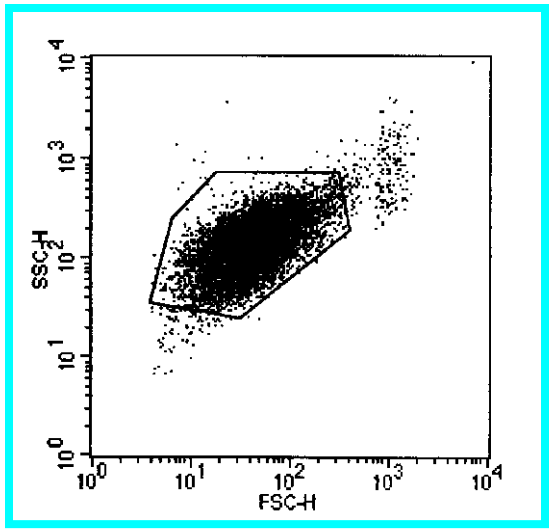


UMBRAL DE FCS

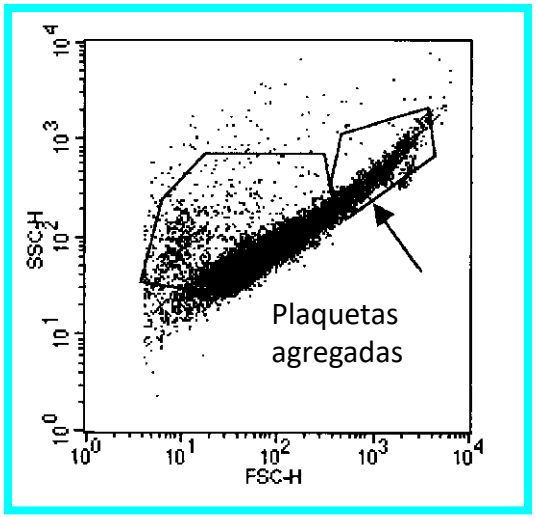


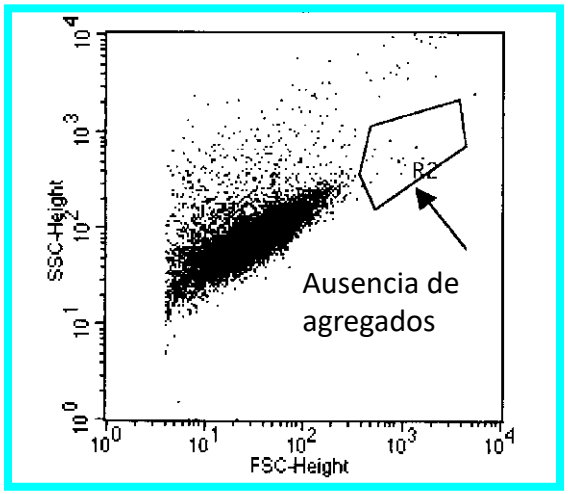
ACTIVACIÓN PLAQUETARIA: CAMBIO DE FORMA Y AGREGACIÓN.

Plaquetas sin estimular



Plaquetas estimuladas con ADP





Plaquetas estimuladas en presencia de un inhibidor de GPIIb-IIIa

CITOMETRÍA DE FLUJO

RECEPTORES DE SUPERFICIE : detección de glicoproteínas de membrana

MARCADORES INTRAPLAQUETARIOS: caracterización de gránulos plaquetarios

MARCADORES DE ACTIVACIÓN: expresión en superficie de glicoproteínas de membranas internas

PRUEBAS DE FUNCIONALIDAD: respuesta a activación con diferentes agonistas, movilización de calcio intracelular, detección de respuesta a antiplaquetarios

DETECCIÓN DE ANTICUERPOS ANTIPLAQUETAS: detección de IgG e IgM asociadas a la membrana plaquetaria

Muchas otras determinaciones …..

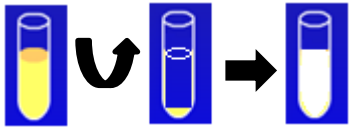
ESQUEMA BÁSICO DE MARCACIÓN

Volumen de muestra: 1-5 mL de sangre
Anticoagulantes: citrato de sodio, ACD, EDTA

Tipo de muestra

Sangre entera

Plasma rico en plaquetas



Plaquetas lavadas

500.000 – 1.000.000 plaquetas
(100-300 .10^3 plaquetas/L)

Trazadores fluorescentes (anticuerpos en condiciones de saturación) +
45 uL medio de marcación (medio de adquisición o medio especial)+
5 uL muestra

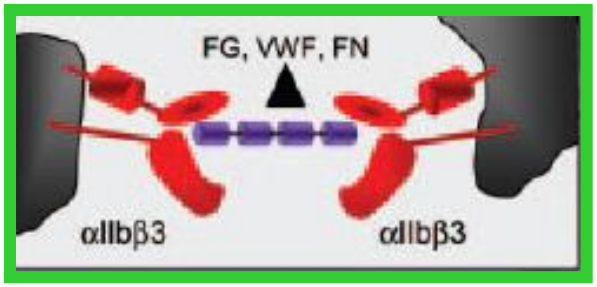
Dilución (medio de adquisición ó medio especial) 500 uL – Dilución y fijación (si lo permite el tipo de marcación, medio de adquisición con paraformaldehído 0.2 %)

Trombocitopatías hereditarias

Anomalías de los receptores para proteínas adhesivas

. GP IIb/IIIa:

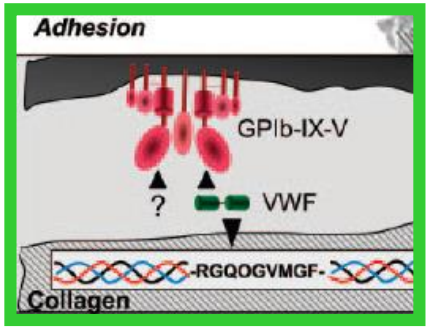
Tromboastenia de Glanzmann



. GP Ib-V-IX:

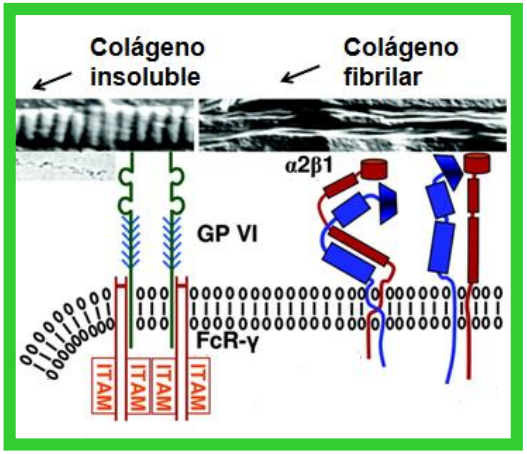
- Bernard-Soulier

- von Willebrand tipo plaquetario

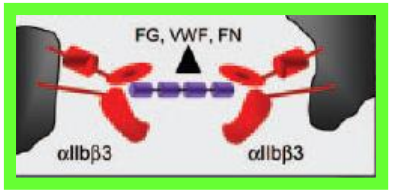


. GP Ia/IIa

. GP VI

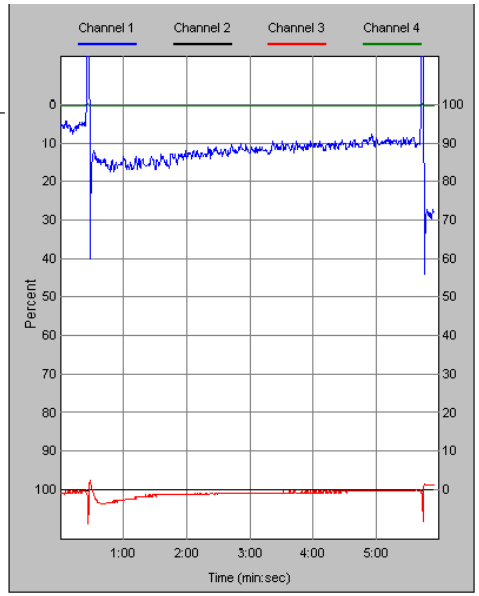


TROMBOASTENIA DE GLANZMANN



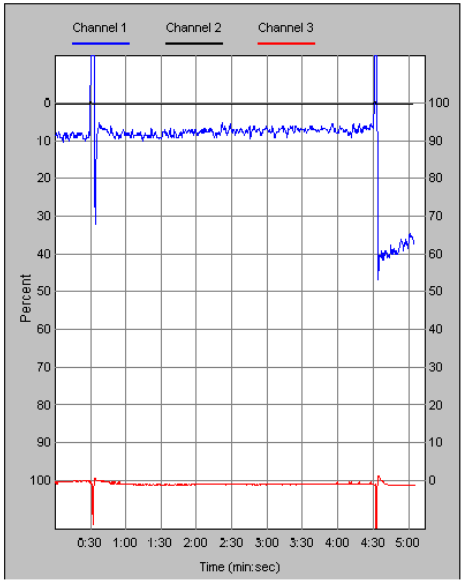
Date **11/09/07** Hosp.

Channel	1	2
Instrument	Lum	Opt
Reagent	ADP 5	
Stirrer	1000	1000



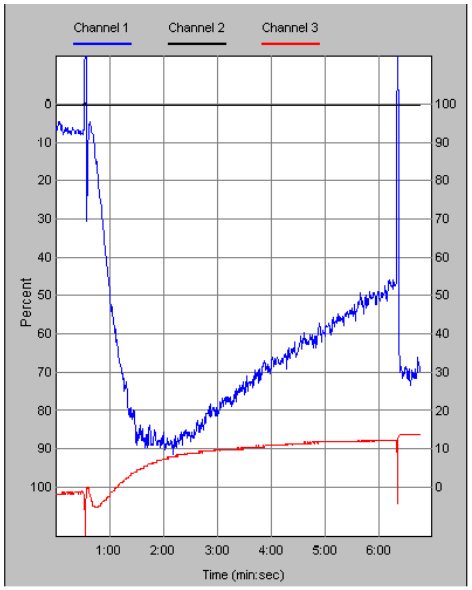
Date **11/09/07** Hosp.

Channel	1	2
Instrument	Lum	Opt
Reagent	ADR 10 uM	
Stirrer	1000	1000



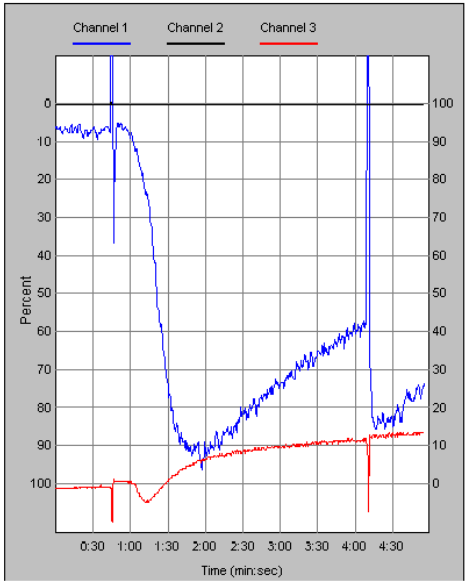
Date **11/09/07** Hosp.

Channel	1	2
Instrument	Lum	Opt
Reagent	AA 0.5 mM	
Stirrer	1000	1000

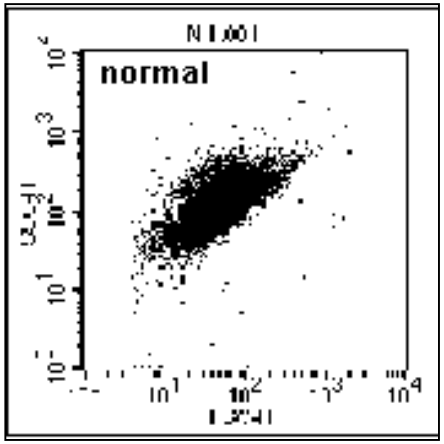


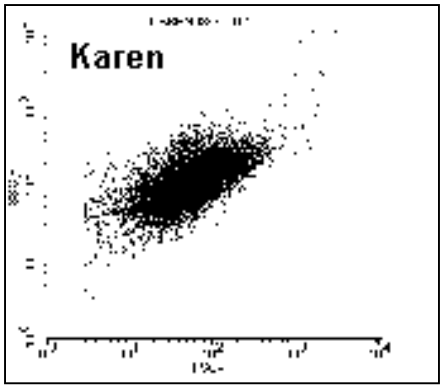
Date **11/09/07** Hosp.

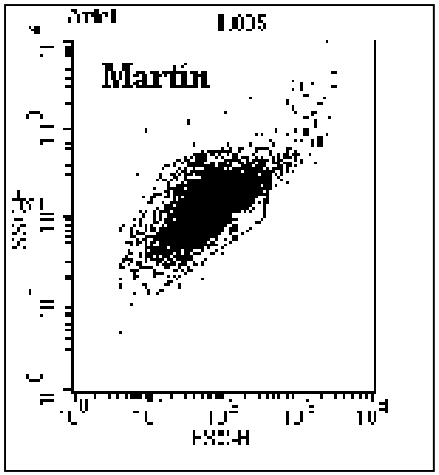
Channel	1	2
Instrument	Lum	Opt
Reagent	COLAGENO 8 ug/ML	
Stirrer	1000	1000

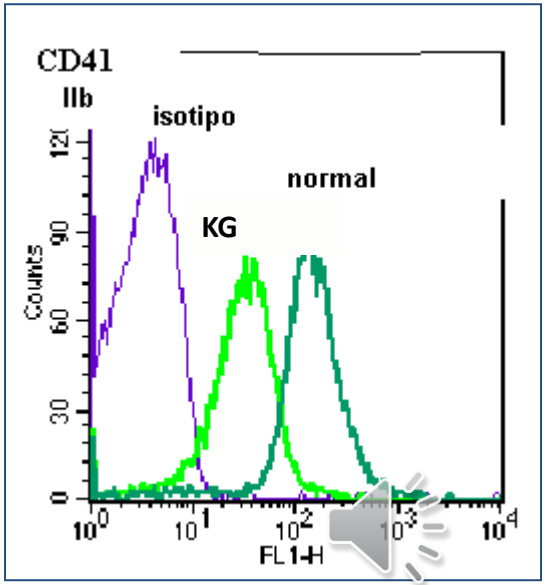


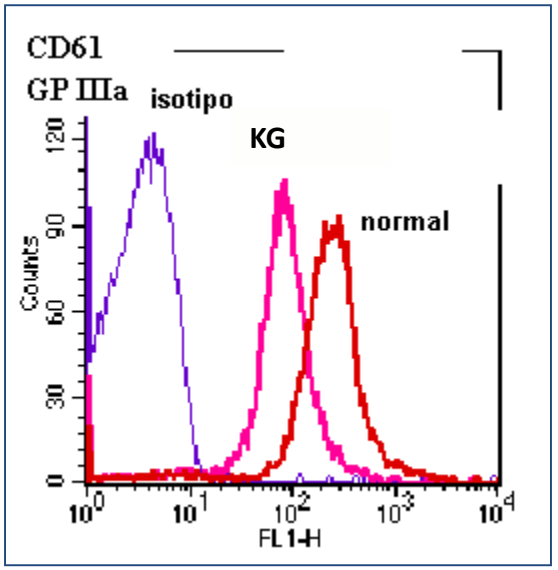
TROMBOASTENIA DE GLAZMMAN: MARCACION EN SUPERFICIE

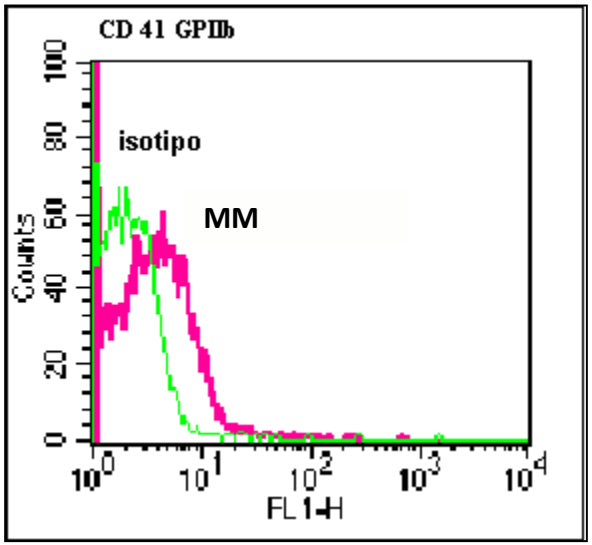


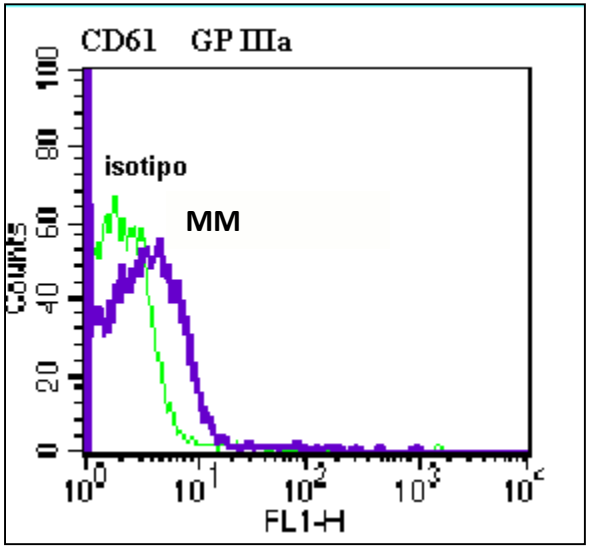




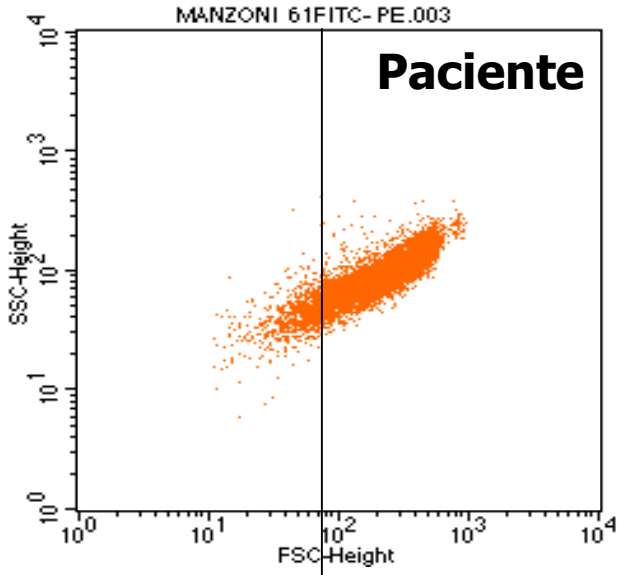


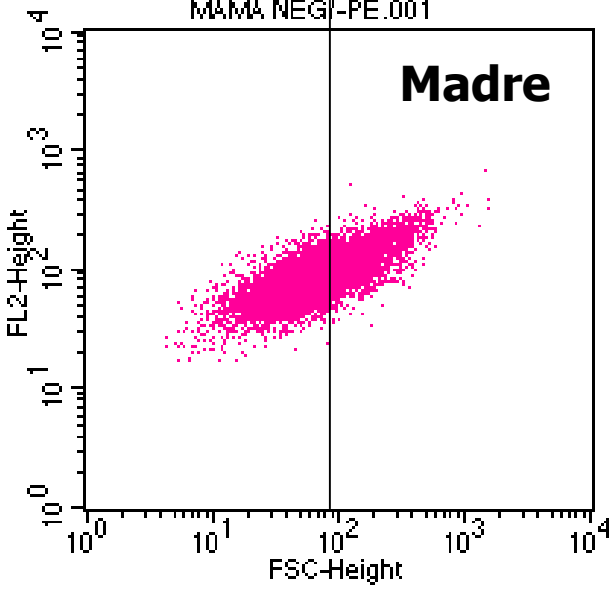


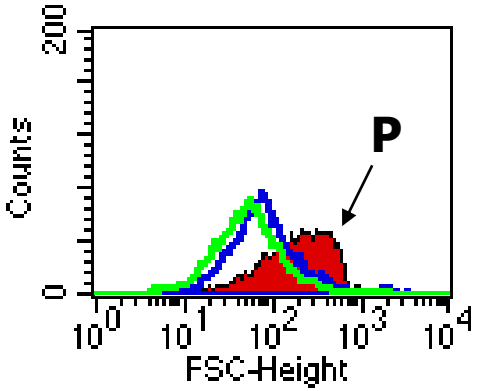




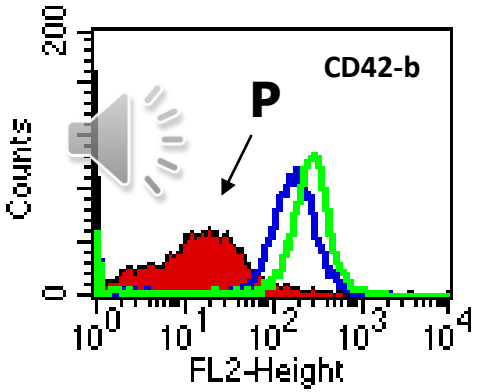
BERNARD SOULLIER



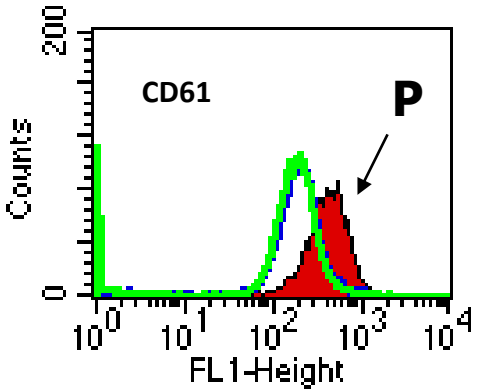




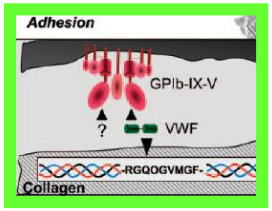
Dispersión frontal- Tamaño (FSH)
P: 202
Madre: 65
Control: 47



CD42-b (GP Ib) (IFM)
P:25
Madre: 171
Control: 257
Relación CD42b/FSH
P: 0.12
M: 2.6
C: 5.5

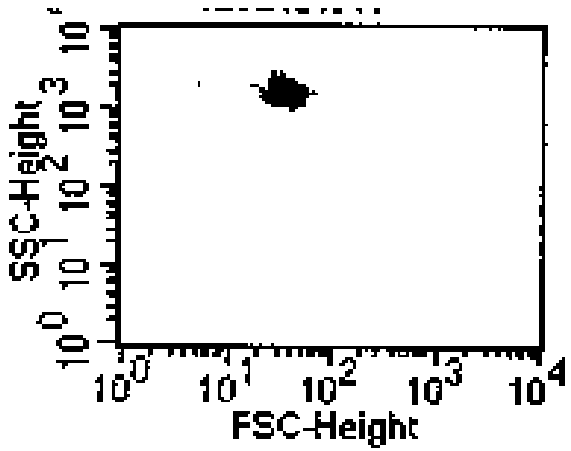


CD61 (GP IIIa) (IFM)
P: 389
Madre: 176
Control: 167
Relación CD61/FSH
P: 1.93
M: 2.7
C: 3.6

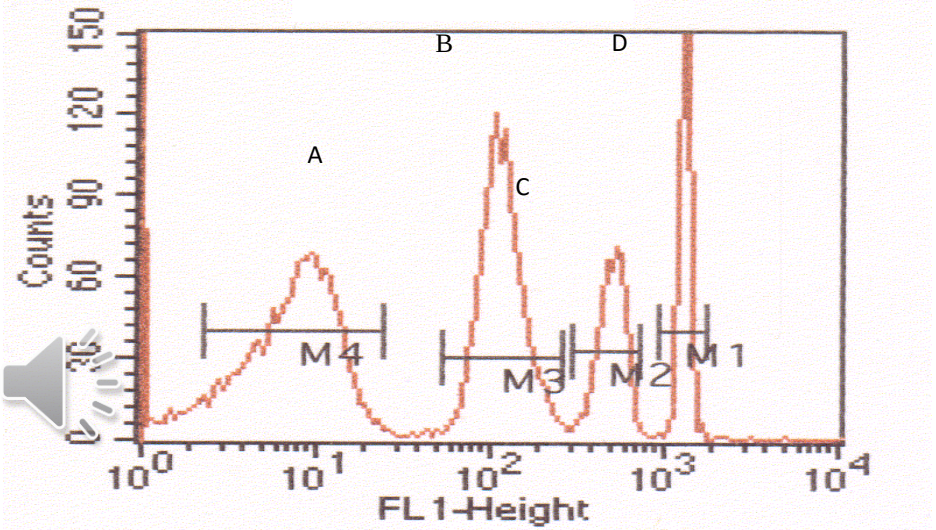


CUANTIFICACION DE GP DE MEMBRANA PLAQUETARIA
CytoQuant™Gp (Stago)

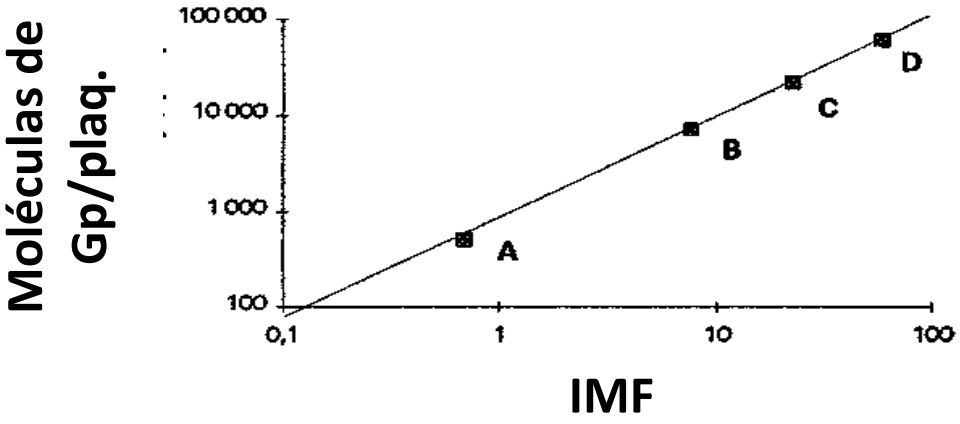
Microesferas: gráfico de puntos FSCH vs SSCH



Histogramas de fluorescencia

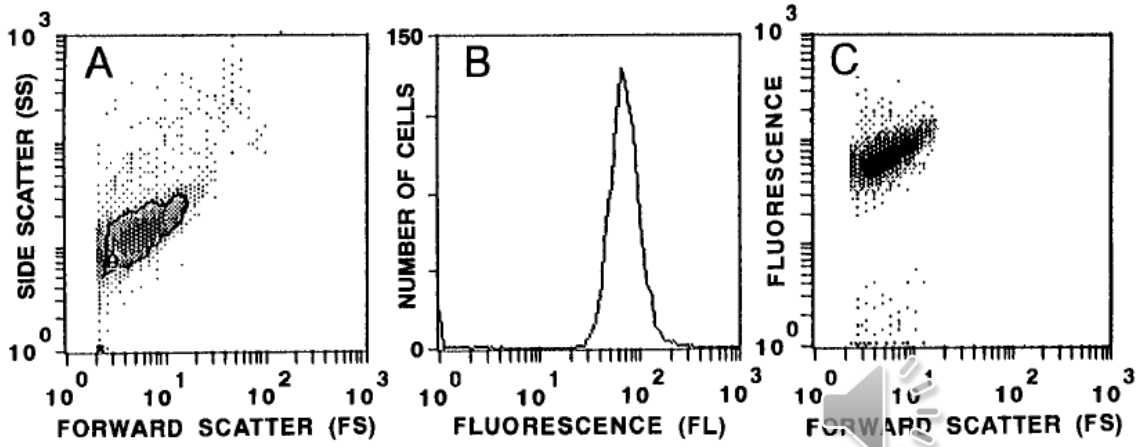


Marker	Media	Nº GP/µesferas
M1 (D)	1201	63000
M2 (C)	466	19000
M3 (B)	112	4500
M4 (A)	8	350

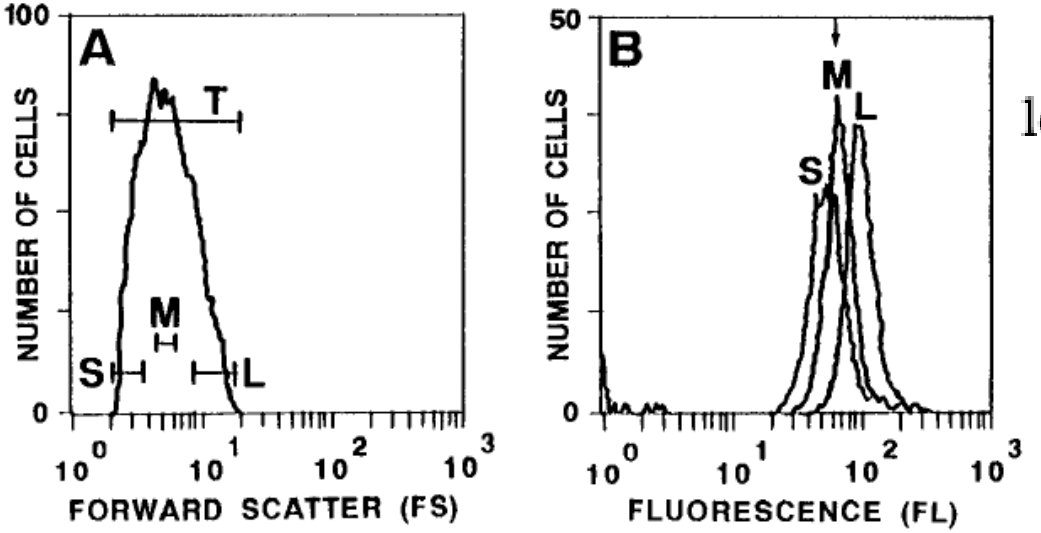


Flow Cytometric Analysis of the Platelet Surface Area and Surface Density of Glycoprotein IIb-IIIa of Unactivated Human Platelets of Various Sizes

V. Leytin,[1] H. Shapiro, I. Novikov,* and J. Radnay



$$D_m = FL_m/FS_m^b$$

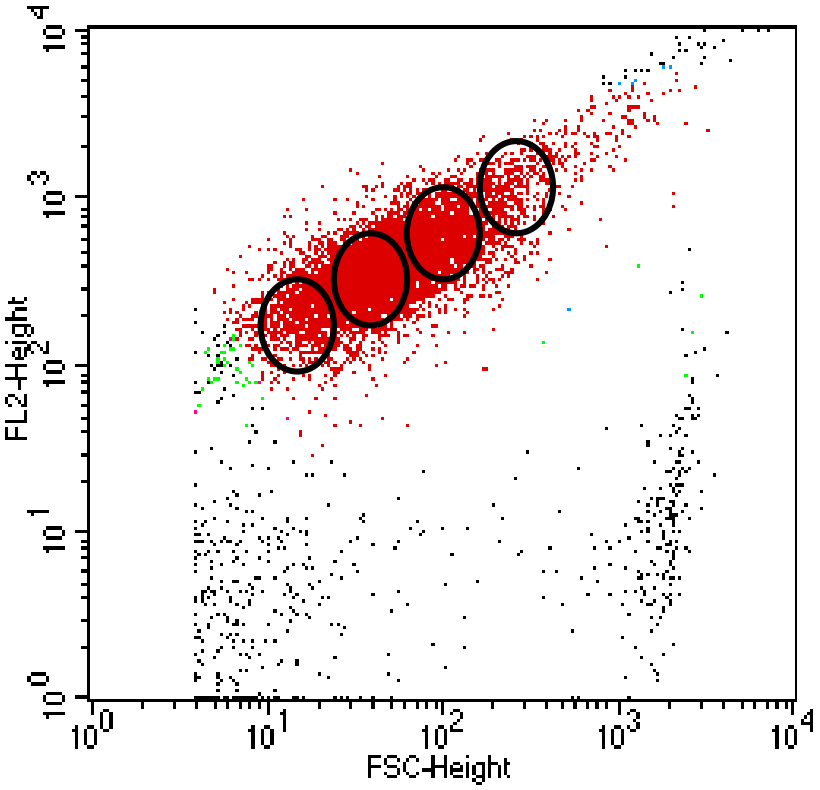


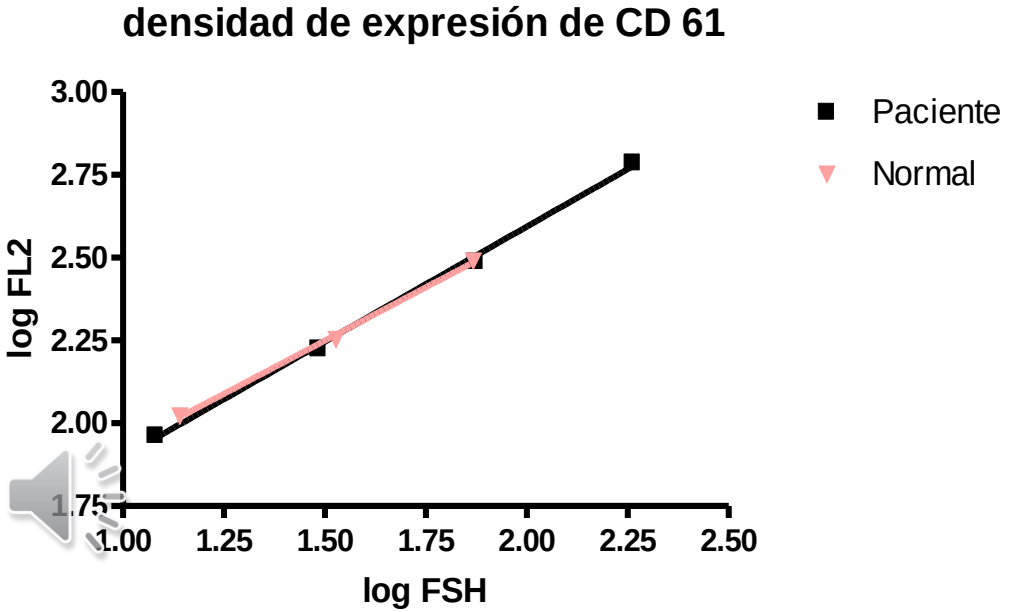
$$\log_{10}FL_m = \log_{10}D_m + b\log_{10}FS_m$$

Leytin V, 1996

Relación de densidades

CD 61 (IIIa)





$$\log_{10}FL_m = \log_{10}D_m + b\log_{10}FS_m$$

	normal	paciente
Best-fit values		
Slope	0.6948 ± 0.01882	0.6429 ± 0.02894
Y-intercept when X=0.0	1.204 ± 0.03258	1.285 ± 0.04456
X-intercept when Y=0.0	-1.733	-1.999
1/slope	1.439	1.555
95% Confidence Intervals		
Slope	0.6138 to 0.7757	0.2752 to 1.011
Y-intercept when X=0.0	1.064 to 1.344	0.7189 to 1.851
X-intercept when Y=0.0	-2.186 to -1.374	-6.697 to -0.7147
Goodness of Fit		
r^2	0.9985	0.9980
Sy.x	0.01657	0.01488

Dn: anti-log 1.204 = 16.0

Dp: anti-log 1.285 = 19.3

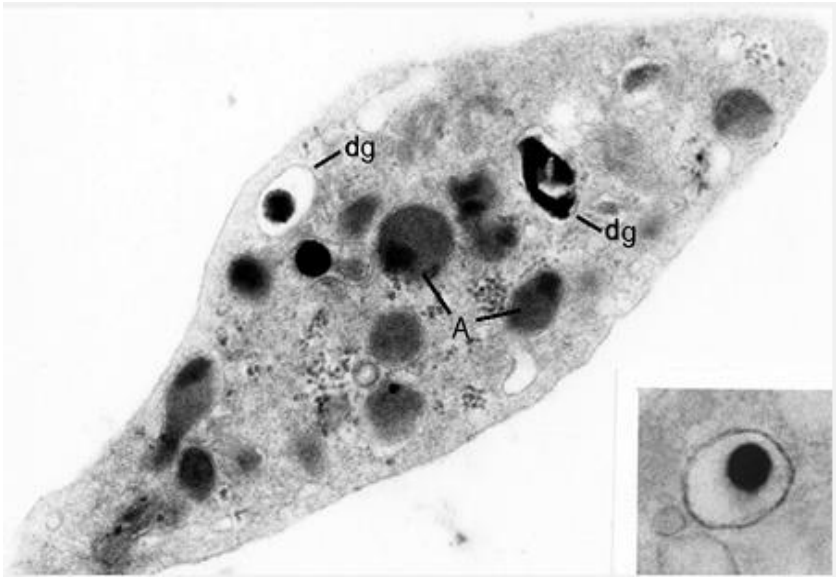
Dp/Dn x 100: 19.3/16.0 x 100 = 120%

Anomalías de los de los gránulos plaquetarios

. Déficit de gránulos densos: déficit de pool de depósito, Hermansky-Pudlak, Chediak Hygashi, TAR, Wiskott-Aldrich.

. Déficit de gránulos alfa: Gray, White, Quebec, Paris-Trousseau-Jacobsen.

. Déficit combinado alfa y densos

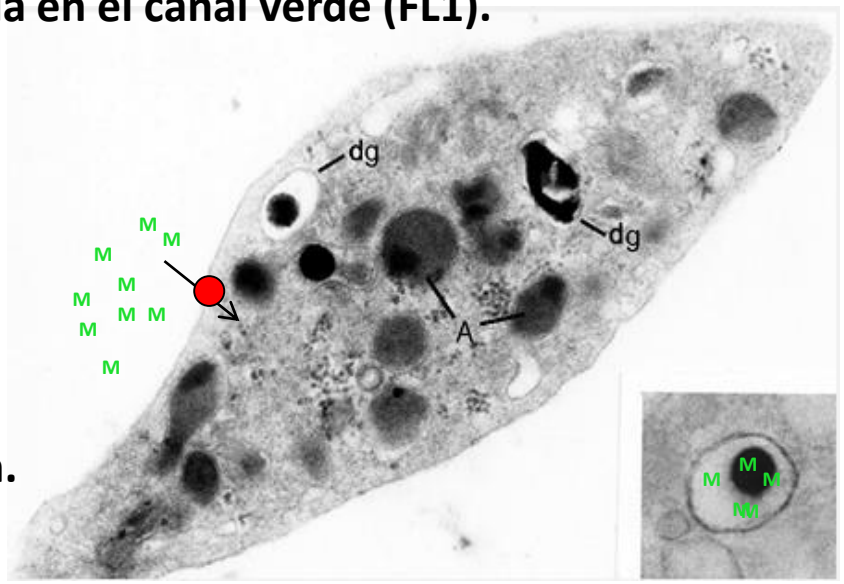


Marcación de gránulos densos (GD) con mepacrina

La mepacrina es un colorante que utiliza el mecanismo de transporte facilitado de serotonina. Tiene afinidad por el ADP y el ATP por lo que quedará retenida en los gránulos densos. Emite fluorescencia en el canal verde (FL1).

Contenido normal de GD: 4-9.

Muestra Plasma rico en plaquetas (PRP) obtenido de sangre anticoagulada con citrato de sodio.
Tampón de marcación (TM): Tris 20.0 mM, 0.15 M ClNa, glucosa 5.0 mM, pH 7.4 .
Mepacrina 5.0 uM diluida en tampón de marcación.



CN
(auto fluorescencia)

500 uL de TM

25 uL PRP

(200.10^9 pl/L)

Mepacrina

500 uL de TM

5.0 uM de mepacrina

25 uL PRP

(200.10^9 pl/L)

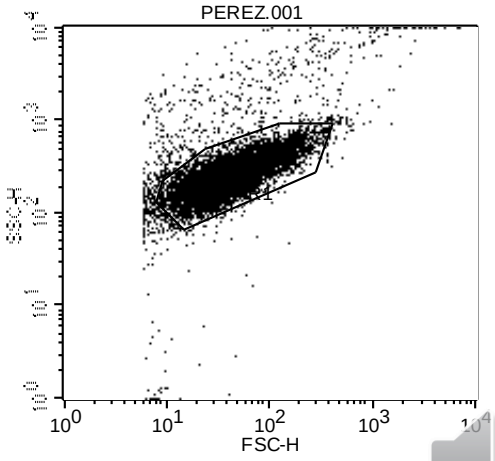
30 min a 37ºC

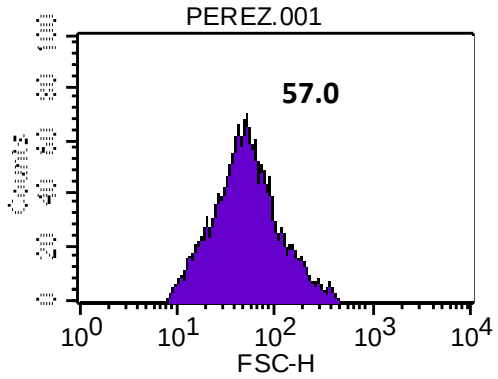
Dilución 1/80 con Isoflow (por ejemplo 12.5 uL de cada mezcla en 1 mL de Isoflow).

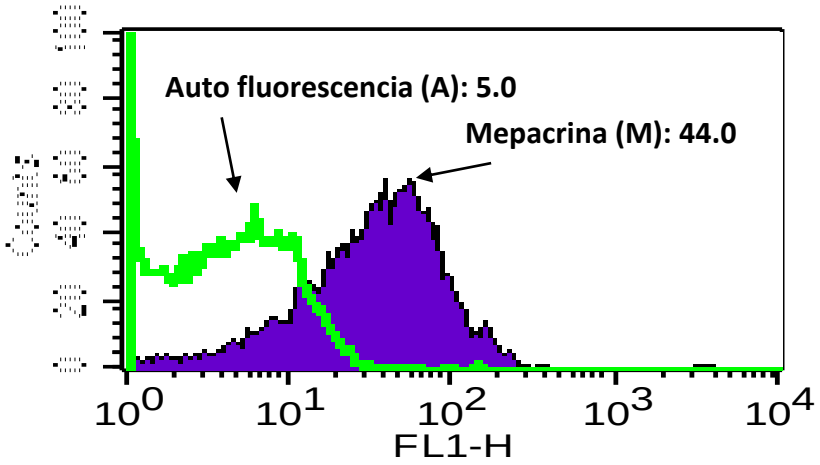
Marcación de GD con mepacrina

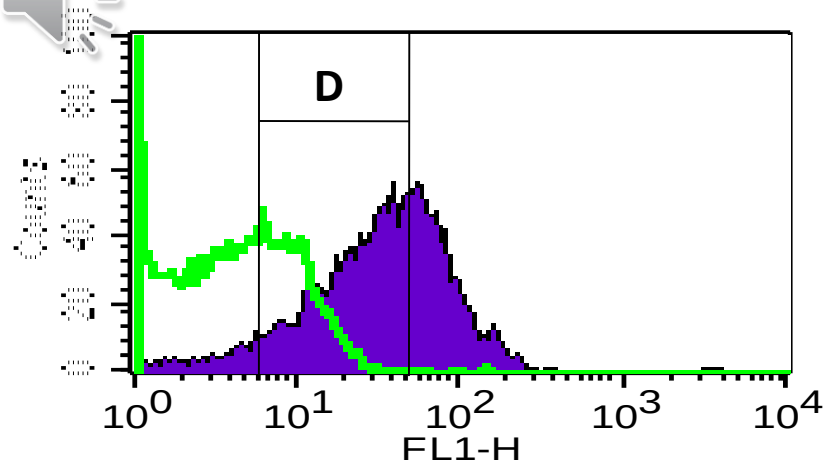
Condiciones de adquisición experimentales:

FSH: EOO Log
SSH: 387 Log
FL1: 550 Log





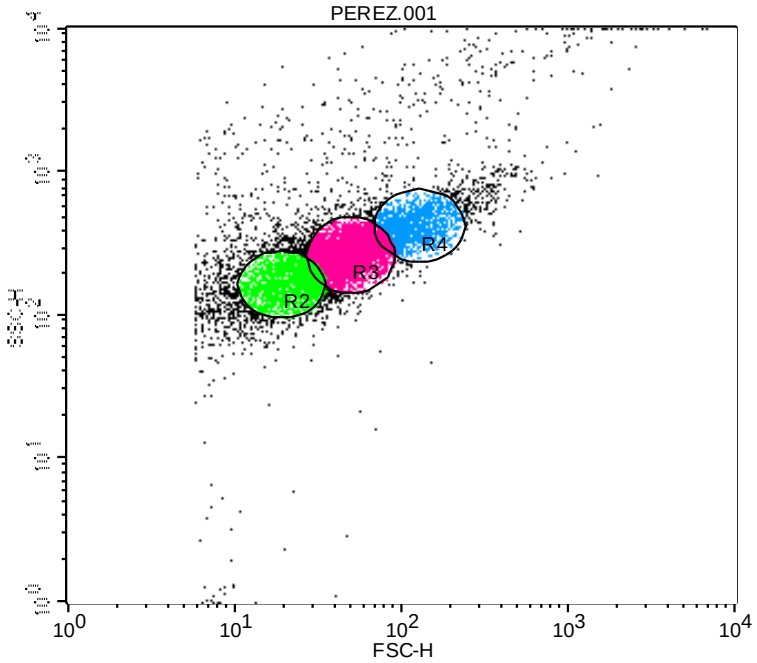


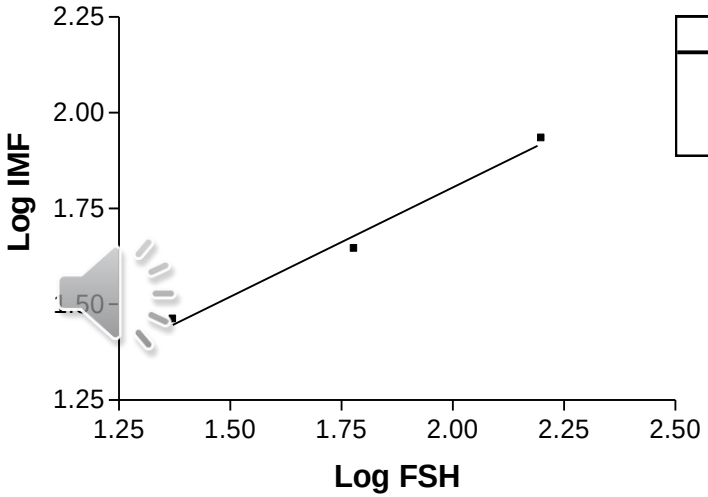


D: constante en diferentes condiciones de adquisición de FL1

D= Log M - Log A = Log M/A

Marcación de GD con mepacrina

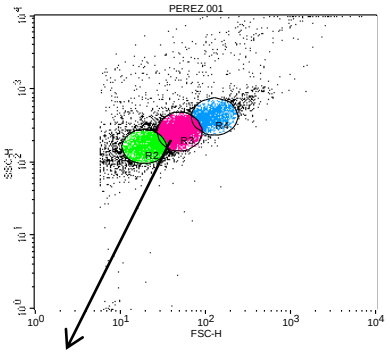


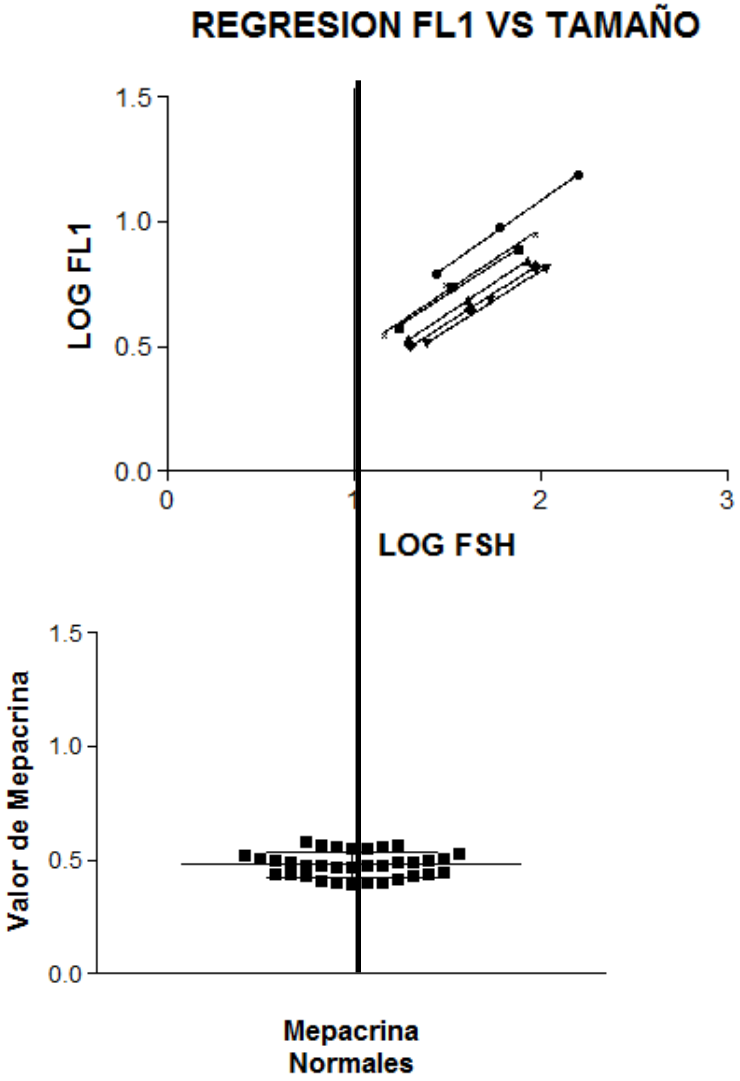


Log FSH	Log IMF
1.371	1.462
1.778	1.646
2.199	1.934

Existe una correlación lineal entre el Log del tamaño (FSH) y el Log de FL1.

Marcación de GD con mepacrina
Cálculo del rango normal





Para simplificar el cálculo:

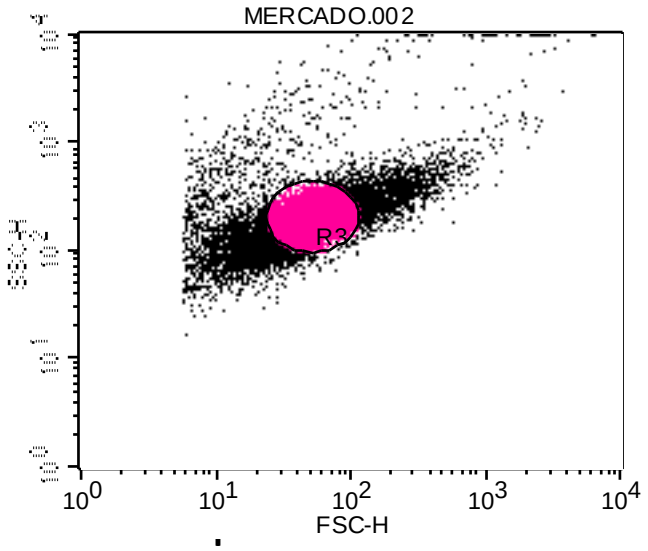
Tomamos como representativa la región central del gráfico de puntos (en este caso R3) obtenemos los valores de FSH y FL1 para A y M.

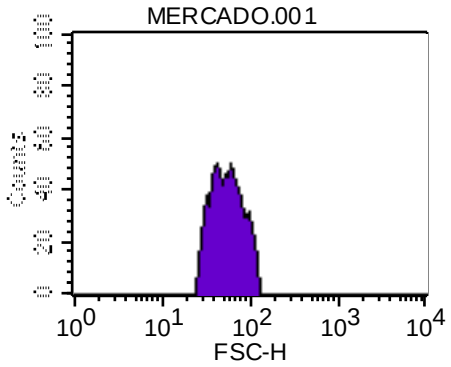
Valor de mepacrina ajustado a tamaño:

Log (FL1 M / FL1 A) / Log FSH

N: 34 individuos normales
Media: 0.48
SD: 0.055
Rango (M+/- 2SD): 0.59-0.37

Paciente 1 con plaquetas de tamaño y número normales:



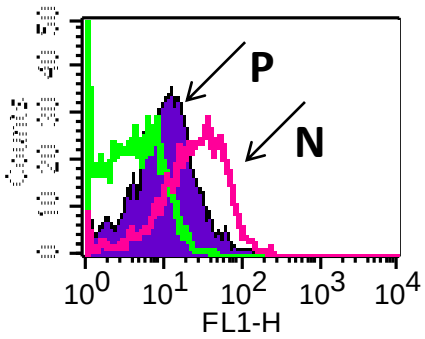


Histogram Statistics

File: MERCADO.001
Sample ID:
Tube: Untitled
Acquisition Date: 09-Aug-16
Gated Events: 4788
X Parameter: FSC-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube I
Gate: G3
Total Events: 10000

Marke	Left, Righ	Events	% Gatec	% Tota	Mean	Geo Mear	CV	Median	Peak Cl
All	1, 99	4788	100.00	47.88	54.36	50.66	38.17	50.03	39



Log (FL1M/FL1A)/ Log FSH= 0.26
DISMINUIDO

Histogram Statistics

File: MERCADO.001
Sample ID:
Tube: Untitled
Acquisition Date: 09-Aug-16
Gated Events: 4788
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube I
Gate: G3
Total Events: 10000

Marke	Left, Righ	Events	% Gatec	% Tota	Mean	Geo Mear	CV	Median	Peak Cl
All	1, 99	4788	100.00	47.88	4.38	3.22	81.82	3.31	1

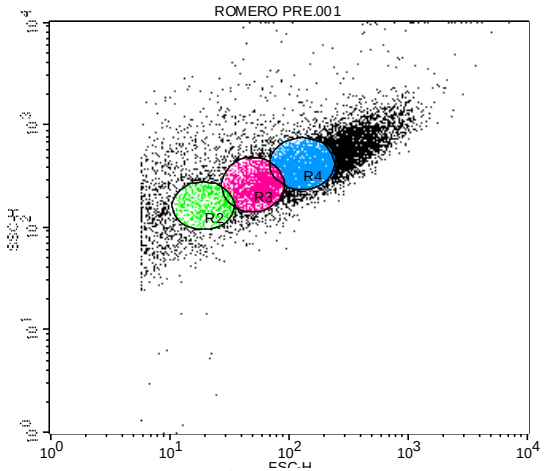
Histogram Statistics

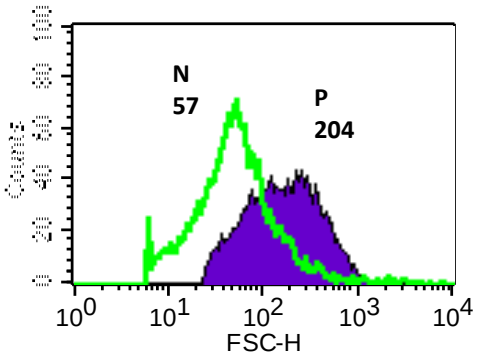
File: MERCADO.002
Sample ID:
Tube: Untitled
Acquisition Date: 09-Aug-16
Gated Events: 5017
X Parameter: FL1-H (Log)

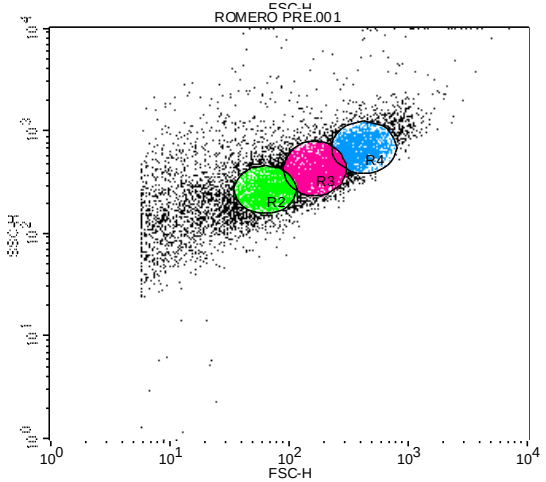
Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube I
Gate: G3
Total Events: 10000

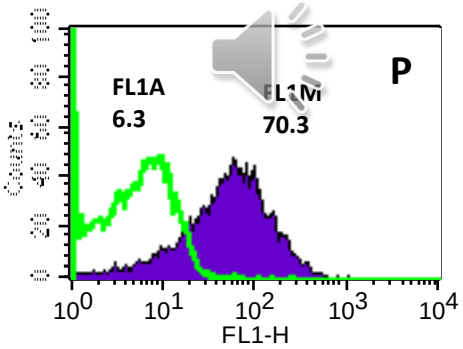
Marke	Left, Righ	Events	% Gatec	% Tota	Mean	Geo Mear	CV	Median	Peak Cl
All	1, 99	5017	100.00	50.17	12.44	9.08	85.03	10.09	1

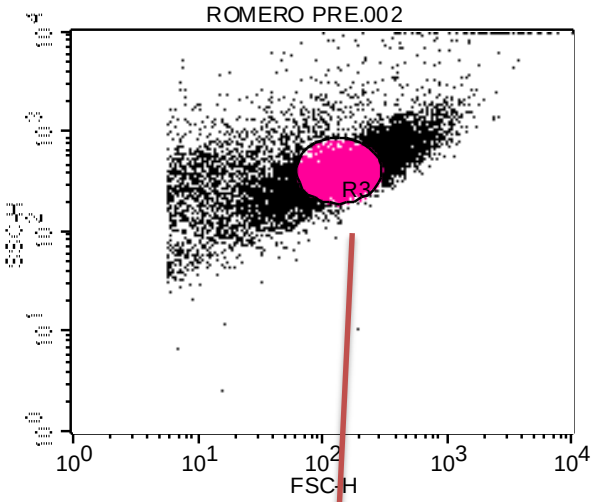
Paciente 2 con macrotrombocitopenia:

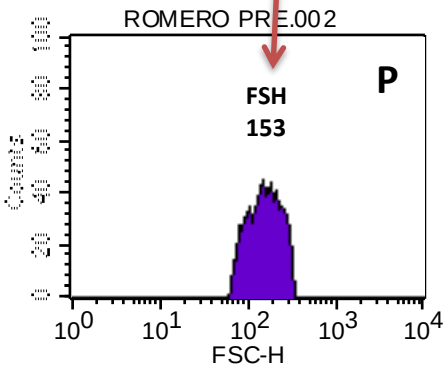


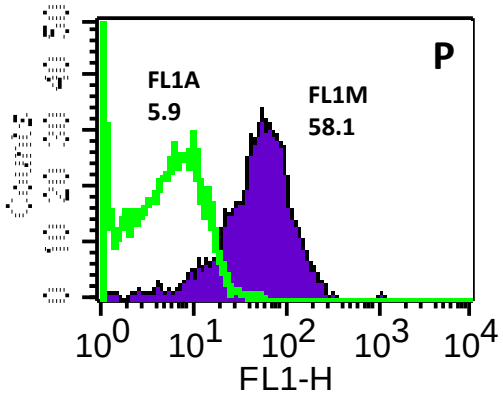






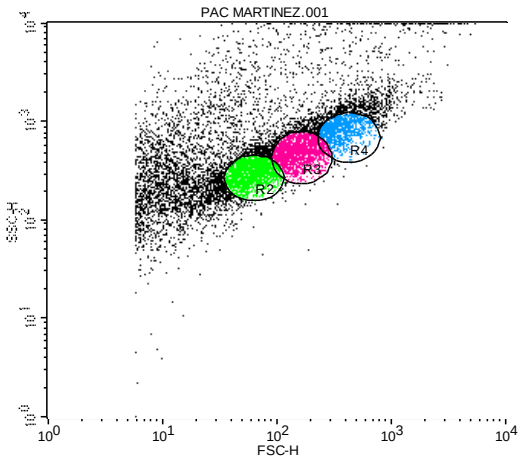


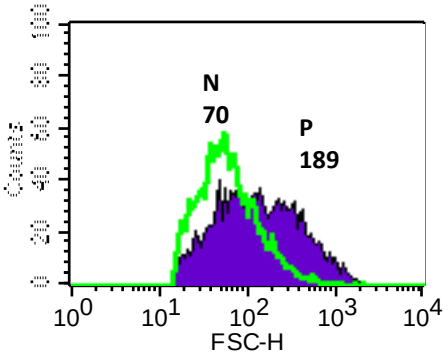


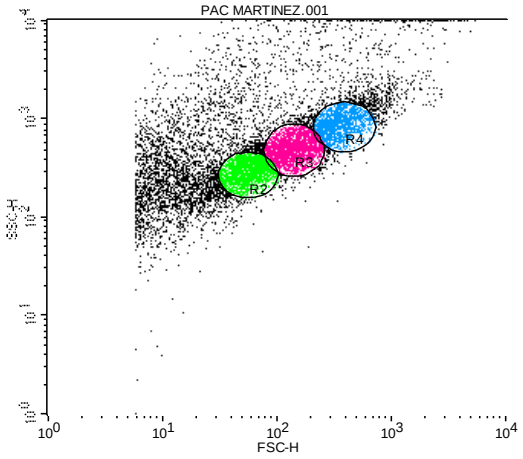


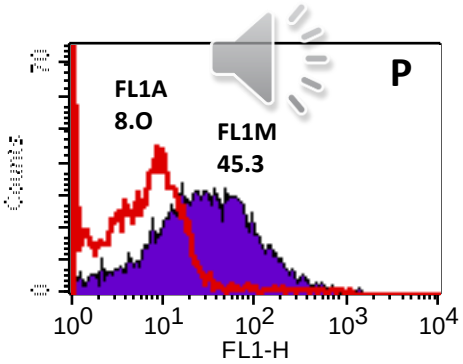
Log (FL1M/FL1A)/ Log FSH= 0.45
NORMAL

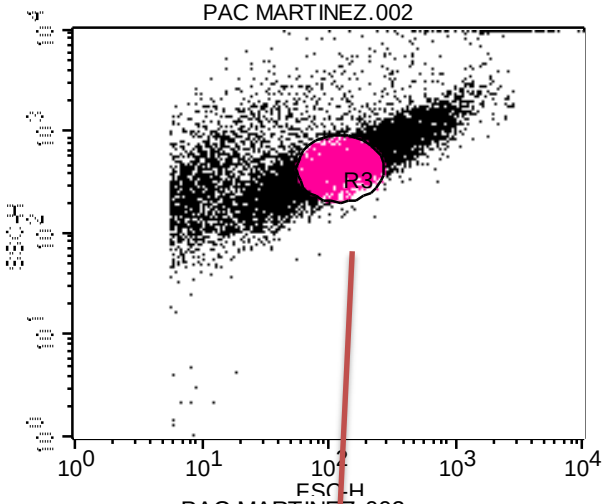
Paciente 3 con macrotrombocitopenia:

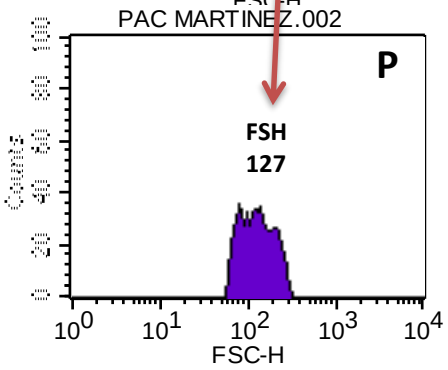


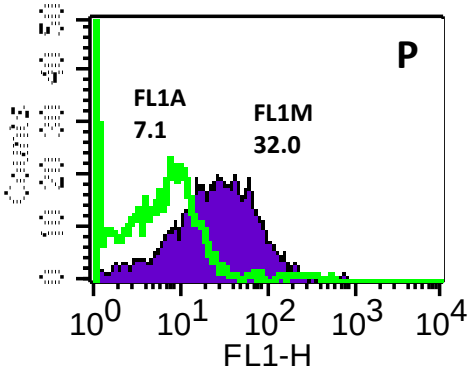












Log (FL1M/FL1A)/ Log FSH= 0.31
DISMINUIDO

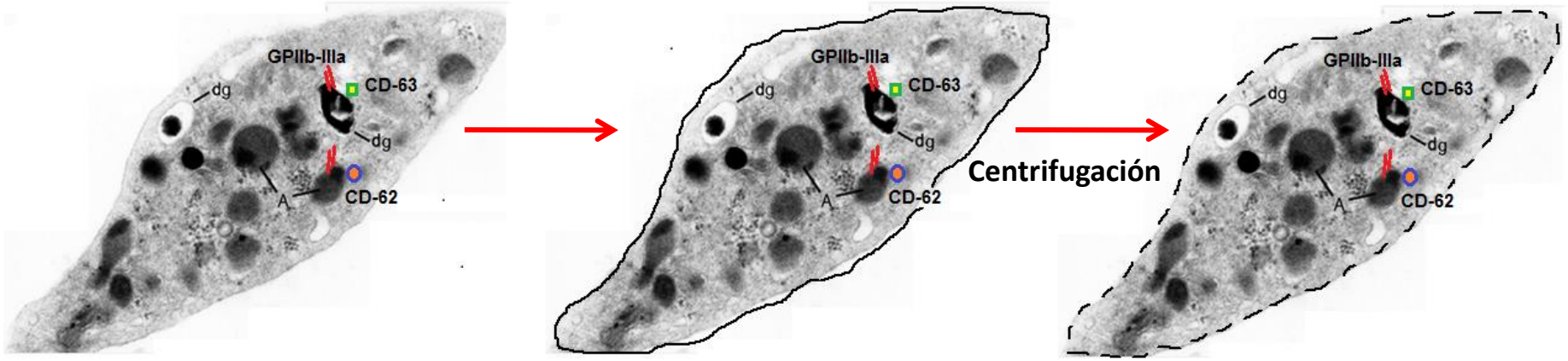
MARCACION INTRAPLAQUETARIA

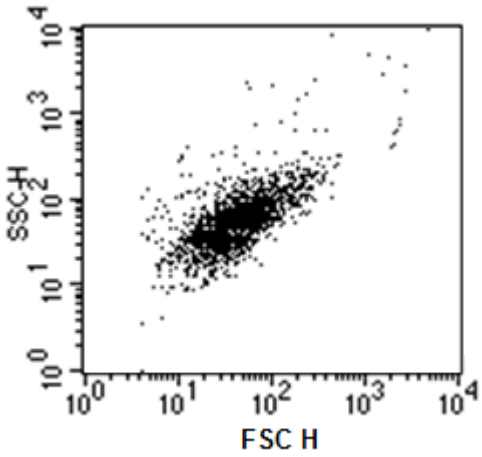
. Proteínas plaquetarias

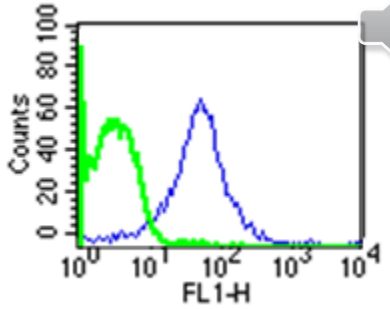
. Plasma rico en plaquetas

Fijación : PRP con PFA 1%

Permeabilización: pellet en solución de saponinas para permeabilización (reactivo comercial) y marcación

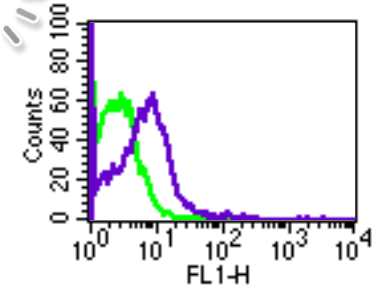




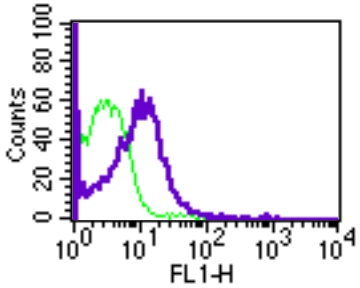


CD-63 (control de Permeabilización)

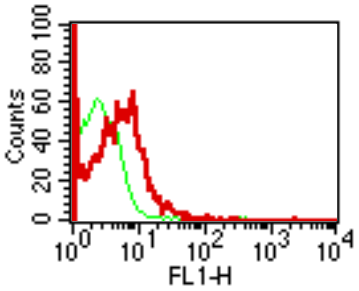
GP IIb en superficie



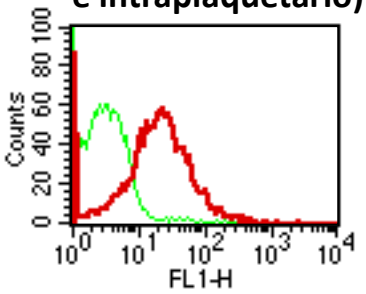
GP IIb total (superficie e intraplaquetario)



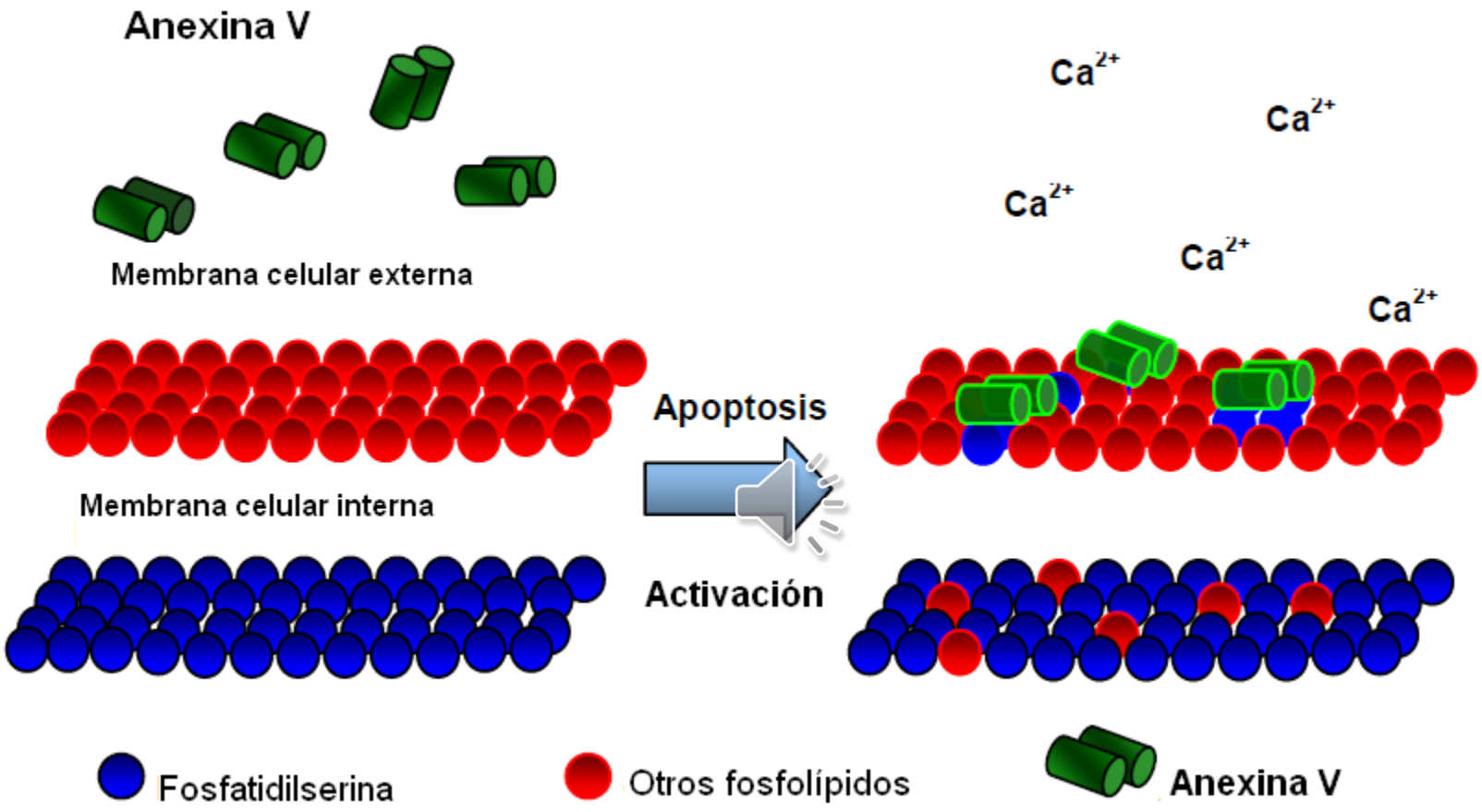
GP IIIa en superficie



GP IIIa total (superficie e intraplaquetario)



Marcación con Anexina V-FITC



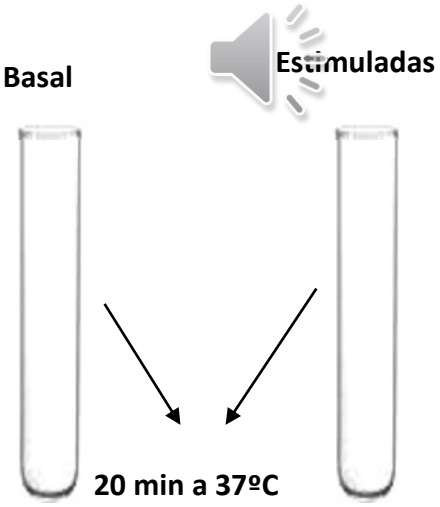
La unión de Anexina V a fosfatidilserina es calcio dependiente, reversible pero fuerte, con una KD: 5.10^{10} M. A baja concentración de fosfatidilserina la relación de unión es de 8 anexinas a una fosfatidilserina.

Condiciones de marcación

Muestra: sangre anticoagulada con citrato de sodio, se trabaja con plasma rico en plaquetas (PRP), centrifugación 5-10 min a 80 g, temperatura ambiente. PRP diluido 1/5 con solución fisiológica estéril (ningún tampón debe llevar conservante).

Tampón de marcación y dilución de muestra: HEPES (10 mM, pH: 7.4 c/NaOH), ClNa 0.15 M, ClK 5 mM, Cl_2Mg 1 mM, Cl_2Ca 2 mM, albúmina bovina 0.1 gr%, glucosa 5 mM. Filtrar con 0.2 uM, alicuotar y guardar a -20 ºC.

Anexina V-FITC: según indicación (Sigma , R&D).
Anti CD41-PE ó CD61-PE: condiciones de saturación.
Inóforo de calcio (Sigma A23187): concentración en tubo de marcación 10 uM.



Tampón de marcación: 100 uL

Anexina-FITC: 1 uL

CD41-PE: 5 uL

PRP dil: 6 uL

Tampón de marcación: 100 uL

Anexina-FITC: 1 uL

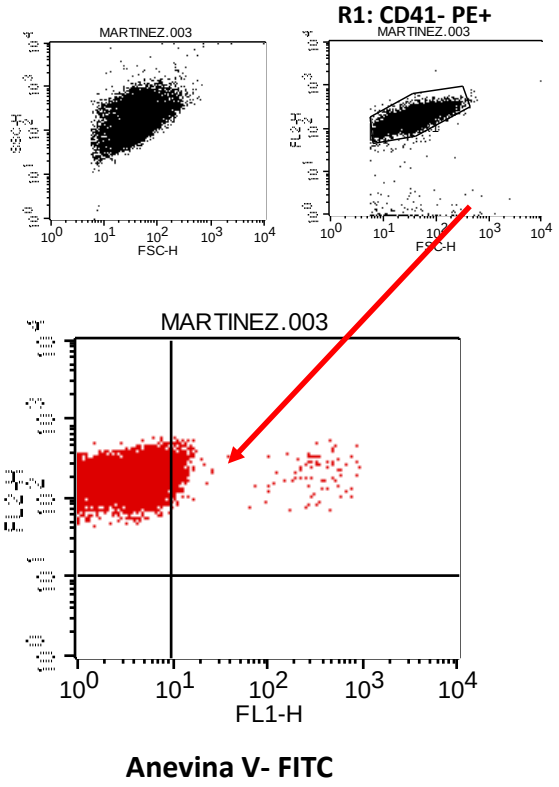
CD41-PE: 5 uL

Inóforo: 1 uL (madre 1mM)

PRP dil: 6 uL

Dilución con 500 uL tampóm de marcación
Análisis al citómetro

Plaquetas sin estímulo



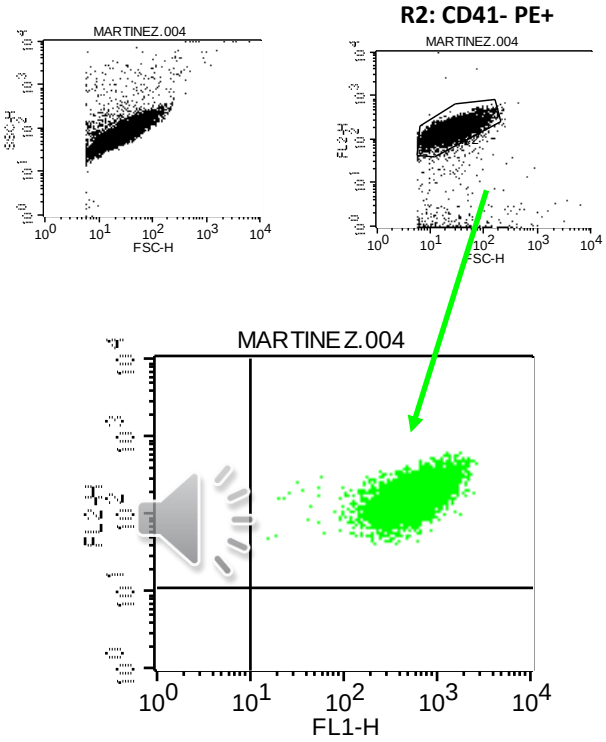
Quadrant Statistics

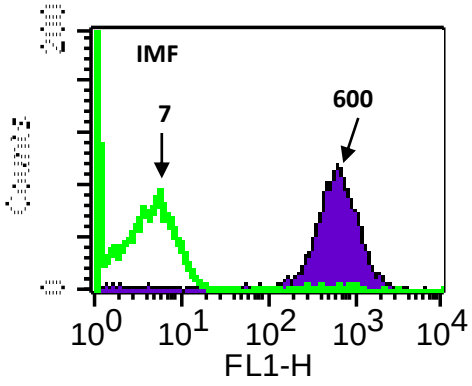
File: MARTINEZ.003
Sample ID:
Tube: Untitled
Acquisition Date: 19-Aug-16
Gated Events: 9781
X Parameter: FL1-H (Log)
Quad Location: 10, 10

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube Lis
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Quac	Events	% Gated	% Tota	X Mean	X Geo Mean	Y Mean	Y Geo Mear
UL	930[illegible]	95.1[illegible]	93.0[illegible]	3.91	3.24	197.0[illegible]	184.4[illegible]
UR	47[illegible]	4.87	4.7[illegible]	65.91	18.87	267.4[illegible]	247.9[illegible]
LL	0	0.0[illegible]	0.0[illegible]	***	***	***	***
LR	0	0.0[illegible]	0.0[illegible]	***	***	***	***

Plaquetas estimuladas con ionóforo 10 uM





Quadrant Statistics

File: MARTINEZ.004
Sample ID:
Tube: Untitled
Acquisition Date: 19-Aug-16
Gated Events: 9441
X Parameter: FL1-H (Log)
Quad Location: 10, 11

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube ...
Gate: G2
Total Events: 10000
Y Parameter: FL2-H (Log)

Quad	Events	%Gated	%To...	X Mear	X Geo Mea	Y Mear	Y Geo Mea
UL	0	0.0[illegible]	0.0[illegible]	***	***	***	***
UR	9441	100.0[illegible]	94.41	599.7[illegible]	538.0[illegible]	189.0[illegible]	175.5[illegible]
LL	0	0.0[illegible]	0.0[illegible]	***	***	***	***
LR	0	0.0[illegible]	0.0[illegible]	***	***	***	***

Marcadores de activación plaquetaria

Table 30-2: Activation-Dependent Monoclonal Antibodies (i.e., Antibodies That Bind to Activated but not Resting Platelets)

Activation-Dependent Platelet Surface Change	Prototypic Antibodies	References
Conformational changes in integrins		
Activation-induced conformational change in integrin αIIbβ3 resulting in exposure of the fibrinogen binding site	PAC1	16
Ligand-induced binding sites (LIBS) on integrin αIIbβ3	PM 1.1, LIBS1, LIBS6	17,101,173
Receptor-induced binding sites (RIBS) on bound fibrinogen	2G5, 9F9, F26	18,32,174
Activation-induced conformation change in integrin α2β1 resulting in exposure of the collagen binding site	IAC-1	175
Exposure of granule membrane proteins		
P-selectin (α-granules)	S12, AC1.2, 1E3	176–178
GMP-33 (α-granules)	RUU-SP 1.77	179,180
CD63 (lysosomes)	CLB-gran/12	181
LAMP-1 (lysosomes)	H5G11	182
LAMP-2 (lysosomes)	H4B4	183
CD40 ligand	TRAP1	184
Lectin–like oxidized LDL receptor-1 (LOX-1)	JTX68	185
Platelet surface binding of secreted platelet proteins		
Thrombospondin	P8, TSP-1	186,187
Multimerin	JS-1	188,189
Development of a procoagulant surface[a]		
Factor V/Va binding	V237	27
Factor X/Xa binding	5224	190
Factor VIII binding	1B3	28

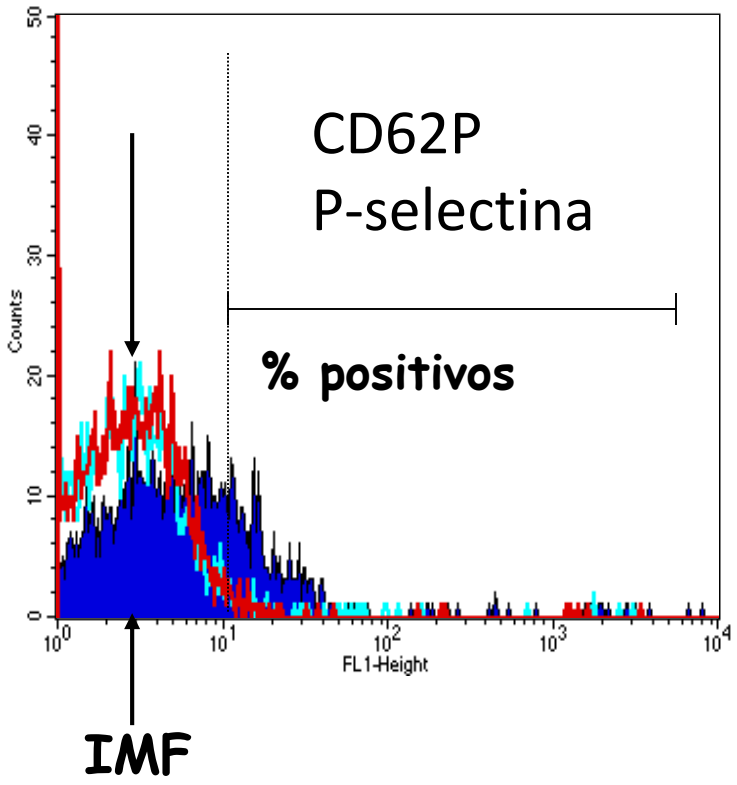
[a]Development of a procoagulant platelet surface can also be detected by the binding of annexin V to phosphatidylserine.[29]

Michelson AD (Platelets, Second Edition)

Aplicación de la citometría de flujo

Marcadores de activación plaquetaria:

- expresión de marcadores de activación en la superficie de la plaqueta (ex vivo)



Condiciones analíticas de difícil estandarización:

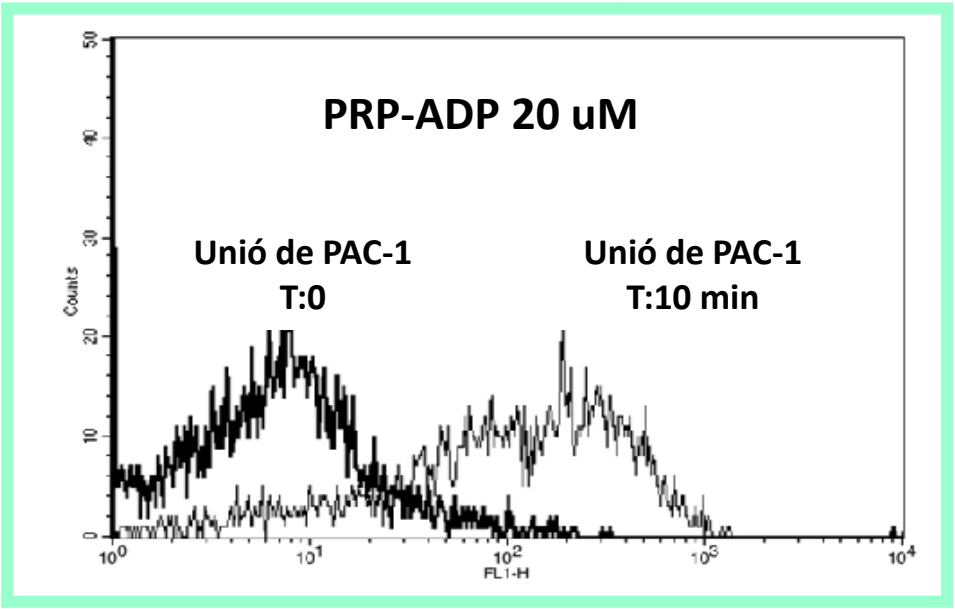
- **anticoagulantes**
- **tiempo de ensayo**
- **controles negativos adecuados**
- **expresión de resultados (% de eventos positivos, intensidad media de fluorescencia)**
- **escasas referencias de CV intra e interensayo**

CINETICA DE UNIÓN

GP de membrana o ligandos: PAC-1, a-FG, a-VWF, GPs

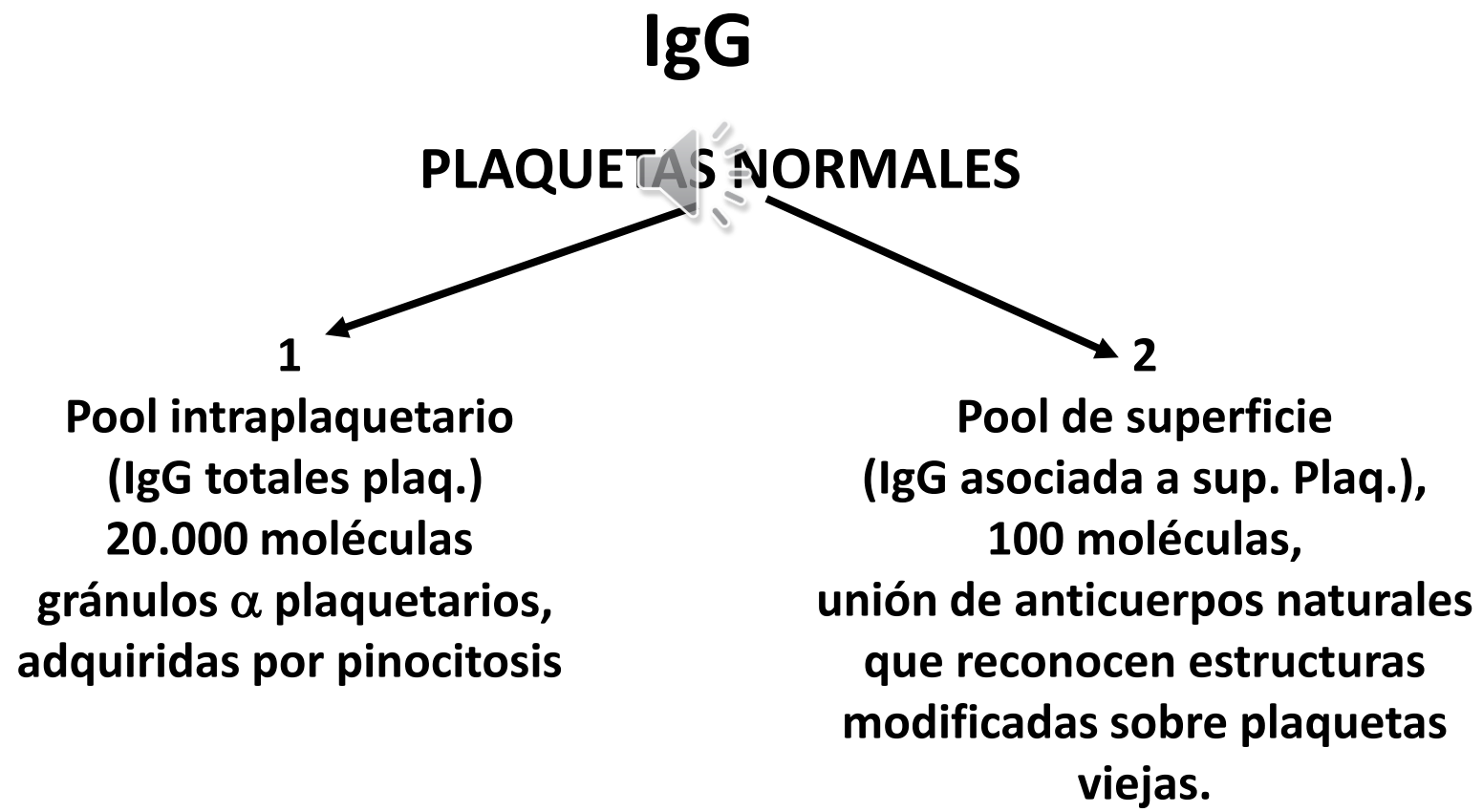
GP de membranas internas: CD62P, CD63

AGONISTAS:
ADP 20μM, Thr 1 a 5 U/ml, TRAP



Inmunoglobulinas asociadas a plaquetas

En trombocitopenias de origen inmune el aumento de la depuración de plaquetas puede estar asociado a la presencia de anticuerpos que reconocen GP de membrana (GP IIb-IIIa, GP Ib, GP VI, GP Ia-IIa).



Inmunoglobulinas asociadas a plaquetas

Aspectos metodológicos

- Obtención del plasma rico en plaquetas

- Lavado de plaquetas

- Anticuerpos: marcadores de población CD 41, CD 42, CD61

-Anti IgG y anti IgM, policlonales, fragmentos $F(ab)_2$

- Control negativo adecuado para medir unión inespecífica

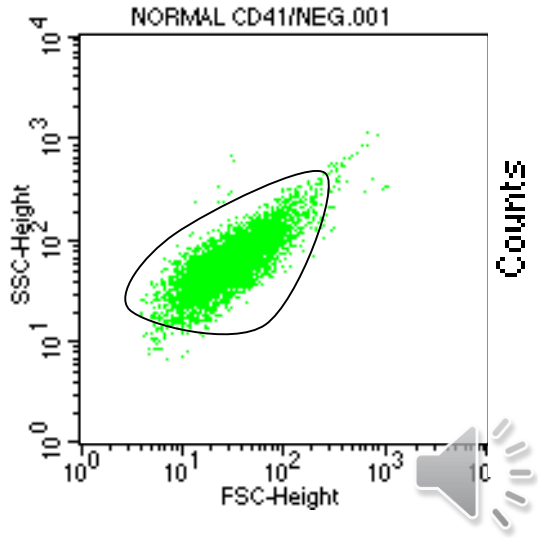
- Control Normal: plaquetas normales procesadas en paralelo

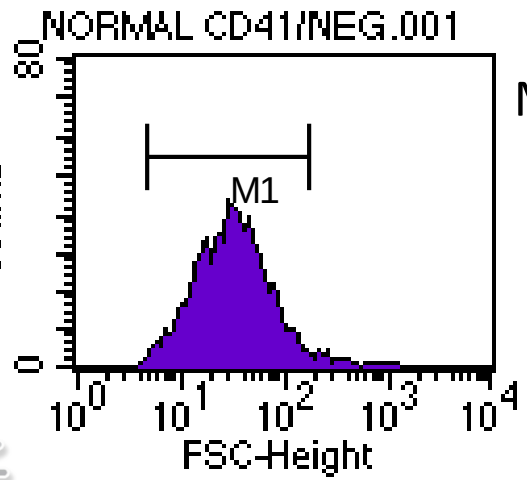
- Expresión de resultados: IFM ajustando a región de FSH constante

- Valores de corte: IgG 11.0 IFM, IgM 6.0 IFM

Inmunoglobulinas asociadas a superficie plaquetaria: detección por citometría de flujo

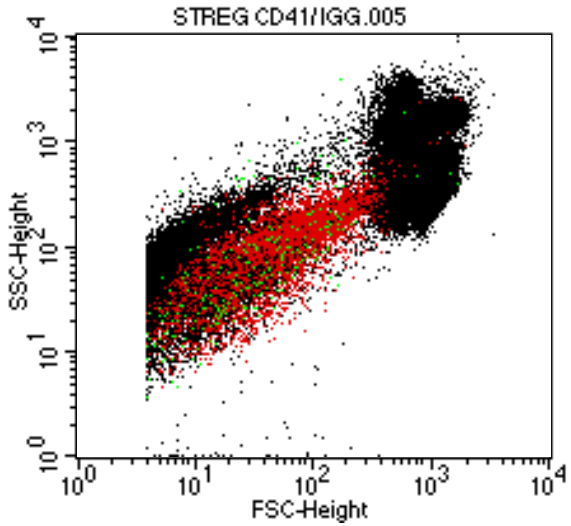
Control normal

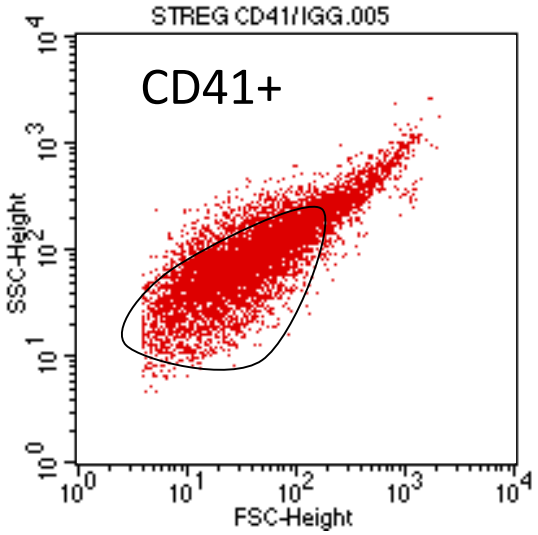


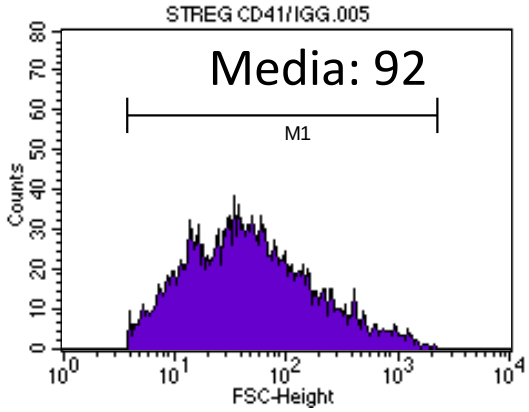


Media: 36

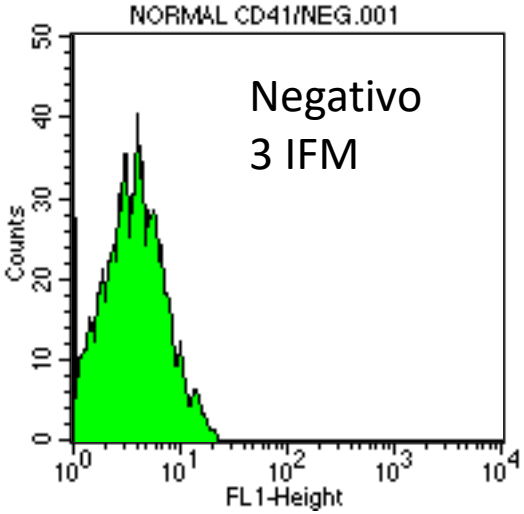
Paciente

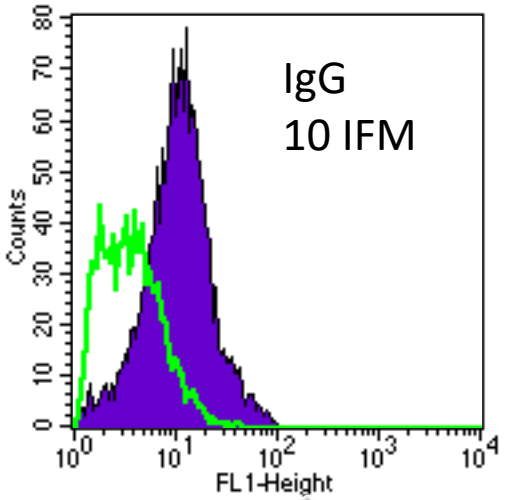


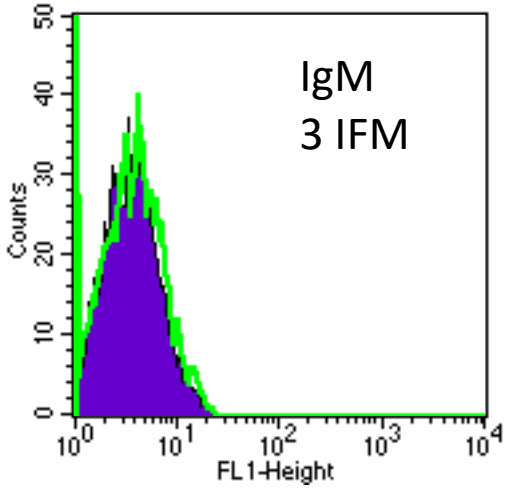




Control normal







Paciente